PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C12N 15/11, 15/74, 15/77, 1/21, 7/01

A1

(11) International Publication Number: WO 94/19460

(43) International Publication Date: 1 September 1994 (01.09.94)

(21) International Application Number: PCT/GB94/00365

(22) International Filing Date: 24 February 1994 (24.02.94)

(30) Priority Data:
9303850.3 25 February 1993 (25.02.93) GB

(71) Applicant *(for all designated States except GB US)*: NORWEGIAN DAIRIES ASSOCIATION [NO/NO]; Breigata 10, N-0187 Oslo (NO).

(71) Applicant *(for GB only)*: DZIEGLEWSKA, Hanna, Eva [GB/GB]; Frank B. Dehn & Co., Imperial House, 15-19 Kingsway, London WC2B 6UZ (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: LILLEHAUG, Dag [NO/NO]; Laboratory of Microbial Gene Technology, P.O. Box 5051, N-1432 Oslo (NO). BIRKELAND, Nils-Kåre [NO/NO]; Laboratory of Microbial Gene Technology, P.O. Box 5051, N-1432 Oslo (NO).

(74) Common Representatives: DZIEGLEWSKA, Hanna, Eva et al.; Frank B. Dehn & Co., Imperial House, 15-19 Kingsway, London WC2B 6UZ (GB).

(81) Designated States: AU, CA, FI, JP, NZ, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: BACTERIOPHAGE ØLC3-BASED VECTOR SYSTEM FOR TRANSFORMATION OF BACTERIA

(57) Abstract

The present invention provides a vector system for introduction of foreign DNA into a bacterial host by site-specific integration, characterized by utilization of phage øLC3 integration functions. More particularly, the invention provides a DNA molecule comprising the nucleotide sequence TTCTTCATG (SEQ ID NO. 1) serving as the core region of attP of phage øLC3 or of the corresponding attB site of a lactic acid bacterium host and a DNA molecule comprising a nucleotide sequence which acts as an integrase promoter and/or encodes an integrase and/or attP of phage øLC3, or a fragment thereof, substantially corresponding to all or a portion of the nucleotide sequence shown in Figure 3 (SEQ ID NO. 2) or a functionally equivalent sequence which is degenerate or substantially homologous with, or which hybridises with any such sequence, as well as vectors containing such DNA molecules.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**GB**	United Kingdom	**MR**	Mauritania
AU	Australia	**GE**	Georgia	**MW**	Malawi
BB	Barbados	**GN**	Guinea	**NE**	Niger
BE	Belgium	**GR**	Greece	**NL**	Netherlands
BF	Burkina Faso	**HU**	Hungary	**NO**	Norway
BG	Bulgaria	**IE**	Ireland	**NZ**	New Zealand
BJ	Benin	**IT**	Italy	**PL**	Poland
BR	Brazil	**JP**	Japan	**PT**	Portugal
BY	Belarus	**KE**	Kenya	**RO**	Romania
CA	Canada	**KG**	Kyrgystan	**RU**	Russian Federation
CF	Central African Republic	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CG	Congo			**SE**	Sweden
CH	Switzerland	**KR**	Republic of Korea	**SI**	Slovenia
CI	Côte d'Ivoire	**KZ**	Kazakhstan	**SK**	Slovakia
CM	Cameroon	**LI**	Liechtenstein	**SN**	Senegal
CN	China	**LK**	Sri Lanka	**TD**	Chad
CS	Czechoslovakia	**LU**	Luxembourg	**TG**	Togo
CZ	Czech Republic	**LV**	Latvia	**TJ**	Tajikistan
DE	Germany	**MC**	Monaco	**TT**	Trinidad and Tobago
DK	Denmark	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**US**	United States of America
FI	Finland	**ML**	Mali	**UZ**	Uzbekistan
FR	France	**MN**	Mongolia	**VN**	Viet Nam
GA	Gabon				

Bacteriophage ØLC3-based vector system for transformation of bacteria

The present invention relates to a novel vector system for transformation of bacteria, especially lactic acid bacteria, and in particular to a vector system based upon the bacteriophage ϕLC3.

Many temperate bacteriophages, (that is bacteriophages having both a lytic and a lysogenic life cycle) are known which insert their DNA into the chromosome of their bacterial host through a site specific recombination process. This integrative recombination process is very characteristic, one of its most notable features being that DNA exchange occurs between two specialised, short DNA sequences, attP on the phage, and attB on the bacterial host, to yield two hybrid phage/host regions attR and attL. These contain between them the phage genome. General recombination function(s) are not required, and these features are illustrated best by the site-specific integration and excision of phage lambda (λ).

The integrative recombination reaction is catalyzed by site-specific recombinases identified in several temperate ccliphages (for a review see Leong et al., 1986), but also in temperate phages from several other bacterial genera, such as Haemophilus influenza (Goodman and Scocca, 1989), staphylococci (Ye and Lee, 1989; Ye et al., 1990), and mycobacteria (Lee et al., 1991). The phage family of site-specific recombinases (Int) is a highly divergent group of enzymes, which also includes the recombinases of several other bacterial genetic elements such as certain bacterial transposons and plasmids (Hall & Vockler, 1987; Ouelette & Roy, 1987; Murphy et al., 1985; Mahillon & Lereclus, 1988; Kubo et al., 1988, and regulatory elements of fimbria synthesis

in Escherischia coli (Klemm, 1986), as well as the site-specific recombinase of the yeast 2-μm plasmid (Hartley & Donelson, 1980). The members of the Int family share limited sequence similarity, especially in the C-terminal region (Argos et al., 1986; Poyart-Salmeron et al., 1989). This region contains a domain believed to be catalytic site of Int, responsible for the breaking and joining of DNA strands during recombination (Pargellis et al., 1988).

Generally, the phage and host attachment sites share a short region of homology, the common "core" sequence, within which the strand exchange reaction takes place. In phage λ the core sequence constitutes 15 base-pairs, but the complete λ attP site required for recombination is about 240 base-pairs and carries binding sites for the λ integrase, λ excisionase (Xis), and host accessory proteins (IHF and FIS) (Thompsen & Landy, 1989, Ball and Johnson, 1991a and b).

The phage λ system has been well studied and vector systems, for example for introduction of heterologous DNA into an E.coli host have been developed. However, by virtue of the specificity of the phage-host infection system, the utility of such λ-based vectors is limited to E.coli. In terms of expression of recombinant DNA, coliform hosts are not always the most appropriate, and new expression systems are continually being sought.

Lactic acid bacteria (LAB) have long found utility in many industries, most notably food and brewing, and in recent years their genetic manipulation appears to have become of increasing importance, for example to improve production of certain products eg. bacteriocins, or enzymes important in fermentation processes.

Moreover, in view of their long term acceptance in many food products, ie. their so-called "food grade" status, LAB represent particularly suitable hosts for the expression of heterologous genes. To date, few expression systems for LAB have been described.

Chromosomal integration vectors for LAB have been constructed by cloning fragments of chromosomal DNA or insertion sequences into a plasmid unable to replicate in gram positive bacteria. These recombinant plasmids have been used to stabilise a plasmid-borne proteinase gene in the Lactococcus genome, to inactivate a gene in L.lactis by replacement recombination, and to introduce heterologous genes into L.lactis and lactobacillus plantarum (see for example Romero et al., 1992; Scheirlinck et al., 1989). The disadvantage of such vector systems, is that they frequently integrate into the chromosome in a head to tail arrangement, and hence are not suitable for studies of gene expression of a single-copy unit.

There is therefore a continuing need for improved vector systems for LAB, and especially for systems for the introduction and expression of foreign genes. The present invention is directed towards providing such an improved vector system.

Temperate bacteriophage infecting LAB are known and lysogeny has been widely reported (see Davidson et al., 1990). Few systems have been characterised however, and for example for Lactobacilli, demonstration of classical lytic and lysogenic cycles of replication have been limited to the phages PL-1, ϕFSW, ϕadh and BKS-T (see Lakshmidevi et al., 1989).

A specific integration vector for Lactobacillus gasseri ADH based on ϕadh has recently been proposed (Raya et al., 1992). There are however, as yet, no reports in the literature on specific phage-based vector systems for lactococci, which tend to be the more important LAB in many food eg. dairy applications.

The present inventors have recently described a novel temperate bacteriophage of lactococci, which has been termed bacteriophage ϕLC3, (Lillehaug et al., 1991). Phage ϕLC3, was isolated from Lactococcus lactis subsp. cremoris after induction with ultraviolet light,

and appears to insert its genome, consisting of a 33-kb double-stranded DNA molecule with cohesive ends, into a specific site in the lactococcal chromosome. We have now characterised the site-specific recombination system of φLC3; the φLC3 attP site has been mapped and sequenced, and a core sequence present in attP, attB, and in the phage-host hybrid regions of φLC3 lysogens (attR, attL) has been identified, together with the φLC3 integrase gene and its promoter.

Accordingly, we now provide, according to the present invention, a vector system for site-specific integration of DNA fragments into the chromosome of bacterial hosts, particularly LAB, characterised by utilization of the phage φLC3 integration system.

More particularly, the core region of attP of φLC3 (and the corresponding attB of L.lactis) within which DNA strand exchange between phage and host occurs, has been identified as the 9 base pair sequence TTCTTCATG (SEQ ID NO. 1).

In one aspect the present invention thus provides the DNA sequence TTCTTCATG (SEQ ID NO. 1) serving as the core region of attP of phage φLC3, and of the corresponding attB of a lactic acid bacterium host.

Viewed from a further aspect the present invention provides a DNA molecule comprising the nucleotide sequence TTCTTCATG (SEQ ID NO. 1) flanked by at least one additional nucleotide sequence such that said molecule is capable of serving either as the attP site of phage φLC3 or as the corresponding attB of L.Lactis.

As used herein, the term "core region" defines the crossing-over region, that is the region within which the DNA strand exchange reaction takes place during site-specific recombination.

The DNA molecule according to the invention may be single or double stranded, genomic or cDNA, and conveniently takes the form of a recombinant DNA molecule.

The additional flanking sequences in the nucleic acid molecule according to the invention may be derived from phage ϕLC3, or the L.lactis host itself or from heterologous sources. In a preferred aspect the flanking sequences may contain one or more restriction sites. Preferably the flanking sequence comprises no more than 2000 bases, more preferably no more than 1000 eg. 500 or 250 base pairs.

As mentioned above, a region of the ϕLC3 genome has been identified and sequenced, which contains the attP site, and, immediately upstream of this, the int gene coding for the integrase enzyme which catalyses the site specific recombination process. Upstream of int is a putative promoter region believed to be the promoter from which the int gene is normally expressed. This sequence is shown in Figure 3 (SEQ ID NO. 2). As will be described in more detail in the Example below, the 1843 bp region shown in Figure 3 (SEQ ID NO. 2) contains a 1.1 kb open reading frame (ORF 374) encoding a 374 amino acid protein believed to be the ϕLC3 site specific integrase (recombinase). Immdiately upstream of this lies the promoter sequence. AttP is located immediately downstream of ORF374, and contains, at nucleotides 1383 to 1391, the unique 9 b.p. core region TTCTTCATG (SEQ ID NO. 1). In particular the attP region has been shown to lie between the end of the int gene and the XbaI site shown in Figure 3 (SEQ ID NO. 2).

Accordingly, a further aspect of the present invention provides a DNA molecule comprising a nucleotide sequence which acts as an integrase promoter and/or encodes an integrase and/or attP of phage ϕLC3, or a fragment thereof, substantially corresponding to all or a portion of the nucleotide sequence shown in Figure 3 (SEQ ID NO. 2) or a functionally equivalent sequence which is degenerator or substantially homologous with, or which hybridises with any such sequence.

Preferably such a DNA molecule comprises nucleotides 1 to 173 of Figure 3 containing the int promoter sequence, and/or nucleotides 174 to 1298 of Figure 3 encoding int, or a functionally equivalent sequence, and/or the attP containing region lying between nucleotides 1299 and 1593 (the XbaI site) of Figure 3, or a functionally equivalent sequence.

DNA molecules according to the invention include degenerate, substantially homologous and hybridising sequences which are capable of coding for the desired activity (int promoter, integrase, attP or attB). "Substantially homologous" as used herein defines sequences displaying at least 60%, preferably at least 70% or 80% sequence identity and also functionally-equivalent allelic variants and related sequences modified by single or multiple base substitution, addition and/or deletion. By "functionally equivalent" is meant nucleotide sequences encoding integrase (int) and/or capable of functioning as the int promoter, the attP site of ϕLC3 or the corresponding attB of a bacterial host.

DNA sequences which hybridise with all or any part of the sequence shown in Figures 3 and 7 (SEQ ID NOS. 2 and 3), or any degenerate, substantially homologous or functionally-equivalent sequences as defined above are also included within the scope of the invention.

"Hybridisation" as used herein defines those sequences binding under the following conditions: prehybridisation at 65°C for 20 minutes in 0.5M sodium phosphate (pH 7.2), 1mM EDTA and 7% (w/v) SDS, followed by hybridisation at 60°C in the same solution for 18 hours and washing at 60°C, with 50mM sodium phosphate (pH 7.2) and 1% (w/v) SDS for 30 minutes, or conditions of higher stringency.

Thus for example, screening for hybridising sequences according to the present invention may take place under the above conditions using as probes the int

gene sequence shown in Figure 3 (SEQ ID NO. 2) (nucleotides 174 to 1298) or the 294 b.p. region between the C-terminal end of the int gene and the XbaI site (nucleotides 1299-1593 of Figure 3) (SEQ ID NO. 2).

As mentioned above, also included within the scope of the present invention are DNA molecules comprising nucleotide sequences capable of functioning as the attB site of L.lactis. Such DNA molecules are defined as comprising the 9 b.p. core sequence TTCTTCATG (SEQ ID NO. 1) and as being capable of interacting with attP of ϕLC3 so as to permit site specific recombination and integration of the ϕLC3 genome.

Such DNA molecules include for example the partial attB sequence of L.lactis subsp. cremoris shown in Figure 7 (SEQ ID NO. 3), and functionally-equivalent substantially homologous and hybridising sequences as defined above. Thus, the partial attB sequence of Figure 7 (SEQ ID NO. 3) for example may be used a probe to screen for hybridising attB sequences according to the present invention, under the above-mentioned conditions. Derivative functionally-equivalent nucleotide sequences according to the present invention may be obtained by using conventional methods well known in the art. These include site-directed mutagenesis, random mutagenesis, or enzymatic cleavage and/or ligation of nucleic acids.

Since the recombination reaction between attP and attB results in integration of phage DNA into the host bacterial chromosome, an insertional vector for integration of foreign DNA into a recipient host cell having an attB site as defined above, could simply comprise phage ϕLC3 containing the desired foreign DNA, or a suitable site, for example a restriction site, for its insertion; the foreign DNA would be integrated, along with phage DNA, into the host chromosome.

More conveniently, vectors comprising the various necessary elements may be constructed using techniques

well known in the art, such as are described for example in Sambrook et al., 1989. Such vectors may take the form of any of the conventional vectors well known in the art, and at their simplest comprise an int coding sequence and/or attP site as defined above.

Thus, according to a still further aspect of the invention, there is provided a vector system for introduction of foreign DNA into a bacterial host by site-spectic integration, characterised by utilization of phage øLC3 integration functions.

More particularly the invention provides a vector for introduction of foreign DNA into a bacterial host, said vector comprising a DNA molecule as hereinbefore defined, together with a site for insertion of said foreign DNA, preferably in operative linkage to an expression control sequence.

A suitable expression control sequence for the foreign DNA will comprise for example translational (eg. start and stop codons) and transcriptional control elements (eg. promoter-operator regions, ribosomal binding sites, termination stop sequences).

Thus, for example, the vector may take the form of a plasmid into which are inserted nucleotide sequences containing int and/or attP as defined above. Additional components of the plasmid vector may include an origin of replication, which for LAB will be a gram positive origin of replication, one or more selectable markers for selection of transformants, and a promoter for the integrase.

Alternative vector systems may include, as mentioned above, phage ϕLC3 itself, modified using standard techniques, to permit insertion of the foreign DNA in non-essential regions of the phage genome, for example between the Xba I and SalI restriction sites as shown in Figure 1.

Alternatively the vector may simply take the form of a DNA fragment as a non-replicatible "module" or

cartridge containing the int and attP sequences together with the foreign gene (and optionally its expression control sequences) which is circularised and ligated together. Such a "module" or cartridge can be used directly to transform bacterial hosts (eg. by electroporation or by protoplast-transformation techniques) and integrate into the host chromosome.

Many plasmids are known in the art which could serve as the basis for construction of vectors according to the present invention. For LAB, the origin of replication of the plasmid, if present, may be selected to be gram-negative, so that the plasmid will not be able to replicate extrachromosally in the host. Suitable plasmids include for example pSA34 described by Sanders et al., 1990 which has a gram-negative origin of replication and carries genes conferring chloramphenicol and tetracycline resistance in gram negative bacteria and erythromycin resistance in gram positive bacteria.

Other suitable plasmids include for example plasmid pVA891 carrying a gram negative origin and erythromycin resistance (Macrina et al., 1983); plasmid pKMPI-E and its derivatives, which are derived from pVA891 by insertion of a temperature sensitive lactococcal replication region (Horng et al., 1991 and Polzin and McKay, 1992); and the pG+ host series of plasmids, commercially available from Appligene Inc., which are derived from a replication-sensitive mutant of the broad-host-range plasmid pGK12 (see Maguin et al., 1992). The pG^{+} vector series are thermosensitive plasmids in which replication can be blocked by increasing the temperature from 30°C to 37°C.

Promoters which may be used in the vectors of the invention include any of the promoters known in the art, including for example the chromosomal, plasmid and phage promoters listed in Table 1 of Guchte et al., 1992. Alternatively the øLC3 promoter from which int is normally expressed may also be used. This is shown in

Figure 3 (SEQ ID NO. 2) at nucleotides 1 to 173.

A wide range of selectable markers are known in the art and described in the literature. Any of these may be used according to the present invention and include for example antibiotic resistance markers as described above. However, properties such as sugar, eg. lactose, utilisation, proteinase production and bacteriocin or bacteriocin resistance may also be used as markers. For a food-grade product, one of such latter markers would need to be used.

A vector system according to the invention may comprise all the necessary elements within a single construct eg. plasmid, modified phage, or "module". Alternatively a single vector may comprise just the int coding sequence (optionally with a promoter) with the attP and foreign gene elements being carried on a separate, complementary, vector. This form of arrangement may be advantageous in certain circumstances. Thus for example, integrase is known to be involved in the phage excision process, and since this is not a feature which is desirable in an efficient integration/expression system, it is advantageous to be able to eliminate the integrase from the host following integration of the desired DNA. This may be achieved for example by having the integrase present separately on a plasmid, eg. a temperature sensitive plasmid, which may readily be cured from the host following integration. In this form of arrangement, the vector carrying int may conveniently comprise a replicon; the vector which carries attP and the foreign DNA does not need to replicate.

The vector according to the invention may be used to introduce DNA into any host bacterial cell carrying a complementary attB site as described above. Such an attB site may be present naturally, for example as in the native host, L.lactis subsp. cremoris, although it has also been identified in several other bacterial

genera. Alternatively, it may be introduced artificially into the host genome, using standard techniques well known in the art, thereby rendering the vector system of the invention generally applicable to all types of host bacteria, including those not naturally susceptible to infection by ϕLC3. Preferably however, the host bacteria are LAB, more preferably Lactococci.

In a further aspect, the present invention accordingly provides a method for introduction of foreign DNA into a bacterial host, said method comprising transforming said bacterial host with one or more vectors comprising a DNA molecule as hereinbefore defined, together with said foreign DNA.

Such transformed host cells form a further aspect of the invention.

The vector system of the invention has a number of uses. Thus, it may be used as a general expression system for genes, encoding desired products, whereby the desired genes are expressed by the transformed cells when grown under suitable culture conditions, following which the desired gene product may be recovered from the cells or culture medium.

As mentioned above, the use of LAB in such an expression system is advantageous in view of the food grade status of LAB, resulting in higher presumed "safety" of products intended for human use.

Foreign DNA introduced and expressed in such circumstances may thus include genes encoding a large range of useful or important proteins or polypeptides, for example enzymes, growth factors, hormones, vaccines or other useful bio-effectors.

As far as vaccines are concerned, the food grade status of recombinant LAB renders them particularly suitable for oral administration of vaccines.

Alternatively the vector system may be used to "improve" bacterial strains used in industrial

processes, for example LAB used in the food industry, particularly the dairy industry, by introduction of desirable characteristics. Thus for example lactococci employed in cheese and yoghurt manufacture may be "improved" by introduction of foreign genes to modify their biochemical pathways and thereby alter the properties or characteristics of the cheese or yoghurt product obtained. For example protease or lactose utilisation enzymes may be introduced, or enzymes involved in diacetyl fermentation, which may have a beneficial effect in the fermentation processes involved in cheese manufacture.

Other genes which may advantageously be introduced to "improve" fermentation processes include autolysis genes, (together with appropriate regulatory elements to prevent premature expression), for example in cheese ripening, where it is desirable to arrest the growth of the fermenting lactococcus.

The vector system may also be used for gene stabilisation. Many useful or important bacterial features, for example traits necessary for successful lactic acid fermentation by LAB, have been found to be encoded by plasmids, and these traits are therefore often unstable due to frequent plasmid loss. One approach for gene stabilisation has been to develop integration vectors able stably to insert genes into the bacterial chromosome. The vectors of the present invention are particularly suited to this purpose.

The characteristics of ϕLC3 integration lend a number of advantageous features to the vector system of the present invention. Most notably the integration of the foreign DNA is always site specific and thus the site of insertion can be strictly controlled, unlike the homologous recombination systems which characterise most conventional vectors and expression systems. This has important implications, particularly from the safety viewpoint. Furthermore it can be guaranteed that

important host genes will not be disrupted. Secondly, a single copy of homologous or heterologous DNA may be introduced by virtue of the site-specific functions. Since a single copy is integrated, expression systems may be performed under conditions that mimic chromosomal genes or operons present in only one copy. Potentially, large fragments of DNA may be integrated, since the att-based recombination system should accommodate at least the genome size of ϕLC3 (33 kb). A further advantage of using a øLC3-based integration system is that efficient integration can be performed without the need for heterologous plasmid sequences. As far as lactococci are concerned, the øLC3 integration apparatus thus could form the basis for an efficient tool for construction of "food-grade" genetically engineered lactococci, with vector sequences derived from a phage normally present in lactococcal starter cultures, and which mediates integration of DNA into a chromosomal site that normally functions as an insertion site.

The invention will now be described in more detail with reference to the following non-limiting Examples, in which:

Figure 1 shows a restriction map of ϕLC3 and a physical and genetic map of the 2.1 kb region carrying the int gene and attP. The nine largest HincII fragments are numbered. The stripped region represents a 1.7 kb region that has been characterised by DNA sequencing (see Fig. 3 (SEQ ID NO. 2)). The restriction sites shown were used for subcloning and mutagenesis. Also shown is the sequencing strategy (pairwise arrows), the location of the probes used in the Southern analysis, the location of the deletions del1 and del2, the location of the int insertion mutations ins1, and the location of coding region for the int gene as well as the location of attP;

Figure 2 shows analysis of the integrated state of phage ϕLC3, localization of the bacteriophage

attachment-site. Southern blots of DNA from the ϕLC3 virion (lane 1-4) and DNA from ϕLC3-lysogenic Lactococcus lactis subsp. cremoris IMN-C18 (lane 5-8), digested with HincII and hybridized with different ^{32}P-labelled ϕLC3 DNA probes. The location of these probes (a, b and c) on the ϕLC3 restriction map are shown in Fig. 1;

Figure 3 shows the nucleotide sequence (SEQ ID NO. 2) of the 1843 bp ϕLC3 DNA region carrying attP, int and the int promoter. The deduced amino acid sequence (SEQ ID NO. 4) of the Int protein is given below the nucleotide sequence. The int termination codon is indicated by an asterisk. Relevant restriction sites are indicated. The putative ribosome binding site (RBS), the core sequence (boxed region), the repeated sequences (R1 to R6) and inverted repeats (R1', R3', R6'), the 20 bp palindrome (P1), and the putative -10 and -35 region of the int-promoter are also indicated.

Figure 4 shows a plot of AT/ATGC from base no. 1000 to base no 1709 in the ϕLC3 attachment region (Fig. 3), using an interval of 100 bases;

Figure 5 shows a comparison of DNA sequences flanking the 9 bp common core (SEQ ID NO. 1) (shown in boldface letters) in the four attachment sites, ϕLC3-(attP) (POP) (SEQ ID NO. 5), the hybrid- (attR (POB) (SEQ ID NO. 6) and attL (BOP') (SEQ ID NO. 7)) and the bacterial attachment-site (attB (BOB') (SEQ ID NO. 8)) and the C-terminal amino acid sequence of a putative gene product of an open reading frame overlapping the attB (SEQ ID NO. 9) and attL (SEQ ID NO. 10) core sequences. The termination codons are indicated by asterisks. Bases in B and B' that are divergent from those in P and P' are shown in lowercase letters. Inverted repeat R6, which overlaps the attB core, is also indicated; and

Figure 6 shows (A) a comparison of the Int proteins of ϕLC3 and L54a. Asterisks and dots indicate identical

and similar amino acids, respectively, the four invariant amino acids are boxed; (B) an alignment of the conserved C-terminal region of ϕLC3 Int with the conserved region of the transposon Tn554 TnpA protein (Murphy et al., 1985) and with the conserved C-terminal region of the Int-family of site-specific recombinases (Poyart-Salmeron et al., 1989). Identical amino acids are indicated by vertical lines. The three invariant amino acids are underlined.

Figure 7 shows the nucleotide sequence of attB and its flanking regions (SEQ ID NO. 3). The 9-base pair attB core region is underlined.

Figure 8 shows a schematic illustration of Int-mediated site-specific recombination between øLC3 attB, located in the chromosome of L. lactis subsp. lactis (AII and BII), and øLC3 attP, located on the pINT1 plasmid (AI), or in the Em^r-int-attP cartridge (BI), resulting in integration of the pINT1 plasmid (AIII), or the Em^r-int-attP cartridge (BIII), respectively. The location and the direction of the EM-resistance-genes are indicated with dotted arrows, the øLC3 int-gene with a shaded arrow, and the temperature-sensitive origin of replication with an open arrow. The location of the øLC3 int-attP module is indicated with a solid box, the core sequence within attP, attB, attL and attR is indicated with an open box. Also shown are the DNA probes used for hybridization (lined boxes) see (Fig. 9), relevant restriction sites, and restriction maps (R.M.) showing the organization of the DNA restriction fragments in the integrated pINT1 plasmid and in the integrated Em^r-int-attP cartridge. The restriction fragments (RF) are labelled in the same way as in Fig. 9. Thin line: bacterial chromosomal DNA, Thickened line: vector DNA + the øLC3 int-attP BamHI-SalI module.

Figure 9 shows Southern analysis of the integrated state of the em^r-int-attP cartridge and of the pINT1 plasmid. Lane 1 and 2: one single loading of a HindIII DNA digest from L. lactis subsp. lactis LMG2230,

containing the EM^r-int-attP cartridge integrated in the bacterial chromosome, sequentially hybridized with the complete ^{32}P-labelled EM^r-int-attP cartridge (EIA) and with the ^{32}P-labelled attB PCR DNA fragment (attB). Lane 3 and 4: one single loading of a HindIII/XbaI DNA digest from L. lactis subsp. lactis LMG2231, containing the pINT1 plasmid integrated in the bacterial chromosome, sequentially hybridized with the complete ^{32}P-labelled pINT1 plasmid (pINT1) and with the ^{32}P-labelled attB PCR DNA fragment (attB). The restriction fragments are named in the same way as in Fig. 1. Bps: number of basepairs. Prior to rehybridization, the probe was removed from the membrane by 30 min. boiling in 0.1X SSC (1X SSC is 0.15 M NaCl plus 0.015 M sodium citrate) - 1% sodium dodecyl sulfate.

Figure 10 shows PCR analysis of the integrated state of the EM^r-int-attP cartridge and of the pINT1 plasmid. The PCR products were amplified from chromosomal DNA extracted from L. lactis subsp. lactis LMG2230 containing the Em^r-int-attP cartridge integrated in the bacterial chromosome (lanes 2 to 4), L. lactis subsp. lactis LMG2231 containing the pINT1 plaasmid integrated in the bacterial chromosome (lanes 5 to 7), or L. lactis subsp lactis LM0230 containing no integrated foreign DNA (lane 8), using øLC3 attB- (lane 2, 5 and 8), øLC3 attL (lane 3 and 6) or øLC3 attR- (lane 4 and 7) specific primers. The figure shows the various PCR products after separation by electrophoresis through a 2% agarose gel. Lane 1 and 9 contains 1-kb DNA ladder (Bethesda Research Laboratories).

EXAMPLE 1

MATERIALS AND METHODS

Phage, bacteria, and plasmids

Phage ϕLC3, isolated from L. lactis subsp. cremoris

after UV induction, has been described previously (Lillehaug et al., 1991). L. lactis subsp. cremoris strain IMN-C18 (Lillehaug, 1992) was used as φLC3 host-indicator strain. φLC3 lysogenic strains were isolated as described by Lillehaug et al., (1991). The Escherichia coli strains DH5α (Hanahan, 1983; Bethesda Research Laboratories) and JM109 (Yanish-Perron et al., 1985) were used for cloning of phage DNA restriction fragments into the vectors pGEM7Zf(+) (Promega Biotec), and M13mp18 and -19 (Norrander et al., 1983), respectively.

Media and Enzymes

Bacteria and phage were propagated in M17 broth or on M17 agar plates (Terzaghi & Sandine, 1975) at 30°C, as described by Lillehaug et al., (1991). Enzymes for lysis of lactococcal cells, mutanolysin and lysozyme, were purchased from Sigma and Boehringer-Mannheim, respectively. DNA modifying enzymes were purchased from Bethesda Research Laboratories. All enzymes were used as recommended by the manufacturer.

Preparation of φLC3 antiserum

Approximately 10^{12} PFU of bacteriophage φLC3, purified twice on a CsCl gradient, was dialysed against 0.9% NaCl, mixed with an equal volume of Freund's complete adjuvant, and shaken to yield an emulsion. 0.5 ml was injected pericutalt into the neck of a rabbit. The rabbit was reinoculated after 4 and 6 weeks, using incomplete adjuvant, and the serum was harvested 2 days after the last injection. For determination of the anti φLC3 titer, 1μl aliquotes of antiserum were added to a dilution series of phage suspended in 1 ml 75 mM $MgCl_2$. After 5 min incubation at 30°C PFU was determined by titration on L. lactis subsp. cremoris strain IMN-C18.

Preparation of DNA

Large-scale purification of ϕLC3 and preparation of phage DNA was done as described by Lillehaug et al., (1991). E. coli plasmid DNA and M13 clones was prepared by standard procedures (Sambrook et al., 1989).

The amount of free phage in a culture of a ϕLC3 lysogenic strain is about 10^{5-6} PFU per ml, indicating a high frequency of spontaneous induction. To reduce the background of free phage DNA in extracts of bacterial DNA, the DNA from the lysogenic strains was extracted from cells grown in M17 broth supplemented with ϕLC3 antiserum. The antiserum, enough to inactivate 10^8 phages per ml of culture, was added to the media immediately prior to inoculation and then to the bacterial cultures every hour during growth. The cultures were incubated until an OD_{600} between 0.5-0.6 was reached, chilled on ice for 15 minutes and then centrifuged at 6,000 x g for 10 minutes. The cells were washed twice in ice-cold TES-buffer (20 mM Tris-Cl pH 7.4, 10 mM NaCl, 5 mM EDTA), and finally resuspended in 1 ml GET-buffer (50 mM glucose, 10 mM EDTA, 25 mM Tris-Cl pH 8.0) which were added 20 mg lysozyme and 500 units mutanolysine per ml. This suspension was incubated on ice for approximately 30 minutes, mixed with 1/2 volume of 10% SDS and carefully shakened at room-temperature for 5 to 10 minutes. Then, 1 volume of chloroform/ isoamyl-alcohol (24:1) was added, and the mixture was seesawed for approximately 20 minutes before the phases were separated by centrifugation. The aqueous phase containing DNA was extracted three times with 1 volume of buffer-equilibrated phenol/chlorophorm (5:1, pH 7.4), each time the tubes were seesawed at room-temperature for several hours. The DNA was precipitated by ethanol, resuspended and in TE (10 mM Tris-Cl pH 7.15, 1 mM EDTA) containing 0.2 μg/μl RNase, incubated at 37°C for 30 minutes, re-extracted once with phenol and precipitated. DNA from non-lysogenic bacterial strains were obtained

as described for the lysogenic strains, except that no ϕLC3 antiserum was added to the bacterial cultures.

The 320-kb bacterial BamHI DNA restriction fragment carrying the ϕLC3 prophage (Lillehaug et al., 1991), used as template for amplification of the attP X attB junction regions, was obtained after digestion and separation through an agarose gel using pulsed-field electrophoresis as previously described (Lillehaug et al., 1991). The fragment was eluted from the agarose gel by using the Geneclean kit of BIO101 (BIO101, La Jolla, CA).

DNA that had been amplified by the polymerase chain reaction was purified by electrophoresis through a 1-2% low melting agarose gel (SeaPlaque GTG agarose, FMC BioProducts, Rockland USA) in TAE buffer (40 mM Tris-acetate, 1 mM EDTA). Prior to the sequencing reaction the electrophorized DNA was either eluted from gel by the Mermaid kit of BIO101 (BIO101, La Jolla, CA) or sliced out of the agarose gel, melted and applied directly to the sequencing reaction mixture.

ss-DNA was prepared from biotinylated ds-DNA by magnetic separation using streptavidinylated magnetic Dynabeads M-280 (Dynal AS, Oslo, Norway). Separation of the two DNA strands was performed as recommended by the manufacturer.

Oligonucleotide primers used for sequencing and/or PCR-amplification were synthesized in an Applied Biosystem 380/381 syntheziser. Biotinylation was done within the DNA synthesizer during synthesis, radiolabeling was performed by [γ-^{32}P]-dATP (Amersham) and T4 polynucleotid kinase (Sambrook et al., 1989).

Recombinant DNA methodology

Transformation of E. coli, agarose electrophoresis, digestion with restriction enzymes and ligation were performed following standard procedures (Sambrook et al., 1989) unless otherwise stated. Prior to cloning of ϕLC3 DNA restriction fragments they were purified by

electrophoresis through an agarose gel followed by elution from the gel by the use of Geneclean.

DNA libraries of the φLC3 host indicator strain, L. lactis subsp. cremoris IMN-C18, were made by ligation of AseI, HindIII, and HpaII DNA restriction digests into the SmaI-, HindIII-, and ClaI-site of pGEM7Zf(+), respectively. The IMN-C18 RsaI restriction fragments were ligated into the SmaI site of the vector after fill-in of the RsaI 5'-protruding DNA ends, by the Klenow fragment of DNA polymerase.

Southern analysis

Electrophorized DNA was blotted from the agarose gel onto a GeneScreen Plus membrane (New England Nuclear, Boston, Mass.) using a LKB vacuum blotter. The depurination (0.25 M HCl), denaturation (0.5 M NaOH, 1.0 M NaCl) and neutralization (1.0 M Tris-HCl [pH 7.0], 1.0 M NaCl) steps were all performed at a vacuum of 45 cm of H_2O for 15 minutes, and the transfer with 10X SSC at a vacuum of 60 cm of H_2O for 90 minutes. The DNA on the membranes was hybridized with φLC3 DNA that had been labeled with ^{32}P using the Nick Translation Systemkit of BRL and [α-^{32}P]dATP (Amersham). The membranes were prehybridized at 65°C for 20 minutes in 0.5 M sodium phosphate (pH 7.2) - 1 mM EDTA - 7% (wt/vol) SDS (Church and Gilbert, 1984). Hybridization was performed at 65°C in the same solution for 16 to 2 hours. After hybridization the membranes were washed three times for 20 minutes at 65°C in 40 mM sodium phosphate (pH 7.2) - 1% (wt/vol) SDS (Church and Gilber, 1984). One membrane was hybridized with several different ^{32}P-labelled probes after dehybridization by boiling for 20 to 30 minutes in a 0.1X SSC - 1% (wt/vol) SDS. The φLC3 restriction fragments used as probes were purified by cloning in pGEM-7Zf(+) before they were labelled with ^{32}P.

Polymerase chain reaction (PCR)

Amplification of DNA by the polymerase chain

reaction technique (Saiki et al., 1988) was done with Taq DNA polymerase using a GeneAmp kit (Perkin Elmer Cetus) and performed in a Perkin Elmer DNA Thermal Cycler. Primers were removed from the PCR reaction mixture by centrifugation through the Centricon-100 microconcentrator (Amicon Div., Grace & Co., MA., USA). The strategies for amplification are shown in Fig. 2.

Before adding the Taq DNA polymerase, the PCR reaction mixture was preheated at 94°C for 5 minutes, to lyse φLC3 viral heads, to inactivate nucleases, and/or denature the DNA. The DNA was amplified by 25 to 35 repeated cycles using various sets of conditions. Denaturation: 1 minute at 94°C, annealing: 30 to 60 sec at a temperature appropriate for each primer, elongation: 1 to 3 minutes at 72°C. Annealing temperatures were selected according to recommendations made by the "Oligo" microcomputer software.

The int-attP region in phage DNA was amplified using DNA from a single φLC3 plaque as template. One plaque was picked from an agar plate by a pasteur pipette and added directly to a 50 µl volume of the PCR reaction mixture at 94°C.

The attP-attB junction regions, attR and attL, were obtained by PCR amplification using the 320kb bacterial BamHI restriction fragment carrying the φLC3 prophage (Lillehaug et al., 1991) as template. Amplification of attR was performed by the "Targeted gene walking" polymerase chain reaction strategy, described by Parker et al. (1991). The attL region was obtained by a modified version of this strategy.

Parker et al. (1991) describe a technique which can be used for amplification of unknown DNA sequences adjacent to a short stretch of known sequence by using a combination of three categories of primers (for details, see Parker et al., 1991): 1) A single, "targeted", sequence specific PCR primer that hybridize to the known target sequence. 2) A set of arbitrary nonspecific "walking" primers. 3) A single, "internal" detection

primer, which is located a short distance downstream with respect to the "targeted" PCR primer. The strategy consists of three consecutive steps: 1) Amplification of arbitrary sequences, using the "targeted" primer and the various "walking" primers. 2) Idendification of target DNA by a PCR oligomer-extension step, using the nested, "internal", detection primer labeled with ^{32}P. 3) Re-amplification and sequencing of target DNA.

The attL sequence was obtained by a modified version of this strategy. By using biotinylated "internal" detection primers at the PCR oligomer-extension step, attL DNA was easily achieved from the PCR reaction mixtures by manetic separation, after removal of free primers and addition of Dynabeads M-280 coated with streptavidin. After sampling, the attL PCR fragments were re-amplified, the PCR reaction mixtures were electrophorized, and the attL DNA excist from the gel and sequenced.

"Targeted" and walking amplification primers for the attR and attL ϕLC3-host junction regions, and nested "internal" detection primers for the oligomer-extension assay are shown in Table 1. The co-ordinates given for the targeted and internal primers refer to their position within the int-attP sequence in Fig. 3 (SEQ ID NO. 2).

The attB region of the non-lysogenic ϕLC3 host-indicator strain, L. lactis subsp. cremoris IMN-C18, was amplified using one biotinylated and one non-biotinylated primer, localized on each side of the common core sequence. The regions further away from the common core was obtained by amplifying attB containing DNA within IMN-C18 DNA libraries. This was done by using one attL or one attR primer, and one primer localized within the vector, after digesting the bacterial DNA with AseI, HindIII, HpaII, or RsaI, and ligation into pGEM7Zf(+).

Table 1

Primer:	Sequence	Position
Targeted PCR and Oligomer-Extension Primers		
attR targeted primer:	5'-AATATATTCGGGATGATTGTGGGA-3'	1629-1606
attR internal primer:	5'-AGTTAAGCCATTCTTCGGAGTGG-3'	1588-1566
attL targeted primer:	5'-CTAGGACACGCAAGTGTGGC-3'	1191-1210
attL internal primer:	5'-AAAATAATTGAGCTGCTACTGGAG-3'	1269-1292
Walking primers:		
W-1	5'-CCTCCGTTAGCTTCTGAAAGTTC-3'	
W-2	5'-GGCGCTTGACAATTCTG-3'	
W-3	5'-GTATTAGCAGCAGCCCCAAAAGCG-3'	
W-4	5'-GAGTATCGATGTATCCATGTCTGAACTAAC-3'	
W-5	5'-GTATATCGATGCGAGCATAATAAACGGC-3'	

Nucleotide sequence analysis

DNA sequence determination was done according to the chain termination method (Sanger et al., 1977) using a Sequenase DNA Sequencing Kit (United States Biochemical) and [α-^{35}S]dATP (Amersham). The primers used for sequencing were the univeral and reverse M13 sequencing primers (Promega) in addition to oligonucleotide primers synthesized in an Applied Biosystems 380/381 DNA synthesizer. Cloned ϕLC3 restriction fragments were sequenced either as double-stranded plasmid DNA (Perbal, 1988) or as single-stranded DNA after subcloning into M13.

DNA that had been amplified by polymerase chain reaction was sequenced directly as double-stranded DNA or as single-stranded DNA prepared by magnetic separation. Direct sequencing of double-stranded PCR DNA was performed by a modified "denaturation by boiling" technique (Perbal, 1988): Prior to the DNA denaturation step, to minimize template renaturation,

the sequencing reaction mixture was either added Nonidet P40 to a final concentration of 0.5% (vol/vol) or low melting agarose (SeaPlaque GTG agarose, FMC BioProducts, Rockland, USA) to a final concentration between 0.5 and 1.0% (wt/vol). The concentration of the sequencing primers ranged from 25 to 50nM. Sequencing of biotinylated ss-DNA after magnetic separation of the two DNA strands, was performed as recommended by the manufacturer. Sequencing the non-biotinylated strand was performed as recommended for M13 ss-DNA by the Sequenase DNA Sequencing Kit (USB).

Directed deletion- and insertion-mutagenesis

Phage φLC3 DNA was first circularized by ligation of the cohesive ends. Removal of the XbaI-SalI fragment and insertion in the BlnI site was done by digestion of the circular DNA with XbaI + SalI and BlnI respectively, followed by Klenow fill-in of the staggered ends and blunt-end ligation (Sambrook et al., 1989). The BlnI-XbaI deletion was constructed in the same way, but because the staggered DNA ends resulting from BlnI and XbaI cleavage are compatible, the Klenow fill-in step was omitted. The ligation mixtures were precipitated with ethanol, resuspended in TE-buffer, and used for transfection of the host strain L. lactis subsp. cremoris IMN-C18 by electroporation. The cells were made competent by growth in 0.3% glycine in the presence of 0.25 M sodium succinate as osmotic stabilizer (Holo & Nes, 1989). The electroporation was performed as described (Holo & Nes, 1989) and immediately following the electric discharge, the suspension was mixed with 0.5 ml of M17 broth supplemented with 0.4% glucose, 0.5 M sucrose, 20 mM $MgCl_2$, and 2 mM $CaCl_2$. After incubation at 30°C for 1 hour 100 µl aliquotes were plated in 0.4% top agar, together with a fresh over-night culture of strain IMN-C18 as indicator, for detection of infective centers. Between 20 and 700 PFU were obtained per µg of

ϕLC3 DNA, varying with different batches of competent cells. All mutants were confirmed by PCR followed by sequencing.

Computer-assisted sequence analysis

Sequence analyses were performed, by use of PCGENE (IntelliGenetics, Mountain View, CA.) microcomputer software. A search for nucleotide and amino acid sequence similarities in the GenBank and Swiss-Prot databases was done by use of the FASTA (Pearson & Lipman, 1988) and the BLAST (Altschul et al., 1990) programs. A comparison of protein sequences was performed by use of the Clustal V program (Higgins et al., 1992).

RESULTS

Localization of the ϕLC3 attP site

Integrative recombination between phage and host DNA generally occurs by a crossover within specific attachment sites located on the phage (attP) and bacterial (attB) chromosome. The strand exchange reaction takes place within a short "common core" sequence present in both attachment sites, and requires a phage-encoded site-specific recombinase (Int). In the integrated state the hybrid phage-host junctions (attL and attR) carry one part of each attachment site. Therefore, a restriction fragment harbouring attP in a digest from the phage particle, will be absent in the digest of prophage DNA. Instead, two new fragments carrying the hybrid phage-host junctions will appear, and the size of these fragments will depend on the DNA sequence flanking the attB site in the bacterial chromosome. Thus, comparison between hybridization patterns obtained when labelled ϕLC3 DNA is used to probe Southern blots of digested DNA extracted from the phage particles and from a lysogenic strain, should

identify the restriction fragment on which attP is located. This strategy has proven useful for mapping of the attP site on the chromosome of several temperate bacteriophages.

At first, Southern analysis of the φLC3 lysogens was confusing. The hybridization patterns of phage and prophage DNA restriction digests appeared to be identical. Leaving out the single cos-fragments, no band disappeared in the lysogens, and no bands which clearly could represent the phage-host hybrid fragments were observed. Later, this was found to be due to a high background of free φLC3 DNA in the lysogenic strain possibly as result of a high level of spontaneous prophage induction. Growth of the cells in the presence of φLC3 antiserum, to avoid a massive and continuous superinfection by the spontaneously released φLC3 phage, reduced the background of free φLC3 DNA, making it possible to identify the various attP, attL and attR carrying restriction fragments. Hybridization of φLC3 DNA to a Southern blot containing phage and prophage HincII digests, then indicated that attP is located on the 1.5 kb HincII fragment no. 7 (Fig. 2). The band representing this fragment was faint in digests from the lysogens, compared to the band in the digest from the phage particle. And furthermore, one new band, possibly representing a phage-host junction fragment appeared. Southern analysis of several other restriction digests, using the entire φLC3 genome and the purified HincII fragment no. 7 as probes, confirmed the presence of attP in this region.

The location of attP within this 1.5 kb HincII fragment was mapped by the use of probes from three different regions of this fragment (Fig. 1, proba a, b and c). Two fragments, present only in the digest from the lysogen, hybridized to probe b (Fig. 2), indicating that this probe hybridizes to both junction fragments (attL and attR) in the lysogen and that attP is located

on this 800 bp HindIII-XbaI probe b fragment. The probes a and c, which flanks the b probe, hybridized to only one of the junction fragments (Fig. 2), clearly demonstrating that φLC3 has integrated into the bacterial chromosome through a site located between the HindIII and XbaI restriction enzyme sites.

The hybridization signal from the attL junction fragment was stronger than that from the attR fragment when hybridized with probe b (Fig. 2), indicating that attP is located quite close to the XbaI site. A φLC3 deletion mutant (delI), constructed in vitro by deletion of the 799 bp XbaI-SalI fragment (Fig. 1), gives rise to stable lysogens, demonstrating that the region essential for integration is located to the left of the XbaI site. The φLC dell mutation does not seem to affect neither lytic nor lysogenic functions.

Southern analysis of various IMN-C18 φLC3 lysogens, and other φLC3 lysogenic L. lactis supsp. cremoris strains, such as the φLC3 parental strain IMN-C3, and an alternative φLC3 host strain, IMN-C17, were all similar, indicating the existence of only one attP site on the φLC3 genome.

Sequence analysis of the attachment site region and identification of the core sequence

The probe b fragment (Fig. 1) on which the φLC3 attP had been localized, was sequenced. For identification of the site at which the recombination between phage and bacterial DNA takes place during integration of phage DNA, the junction regions in a φLC3 lysogenic strain were amplified by the use of PCR, and sequenced. The sequence of the attL and attR regions identified a 9-bp core sequence common to both phage-host junctions, and which also is present in φLC3 DNA (Fig. 5) (SEQ ID NOS 6 and 7). The attB region in L. lactis subsp. lactis strain IMN-C18 was also sequenced after amplification by PCR using primers based on the DNA sequence of the attB arms

of the attL and attR junctions, confirming the presence of the core sequence in the bacterial chromosome (Fig. 5) (SEQ ID NOS 5, 6, 7 and 8). The DNA sequences following and preceding the core in attL, and attR are fully conserved in attP and attB. The crossover between phage and host DNA takes place within this 9-bp common core sequence (SEQ ID NO. 1).

The region surrounding the attP core sequence is relatively rich in A+T, similar to the attachment-sites of other temperate phages (Fig. 4). Several continuous stretches of only A+T are observed in close vicinity to the ϕLC3 core, the longest stretch of 19 bp A+T are located only 21 bp to the right of the core sequence, and the region between the end of orf374 and the XbaI site 290bp further downstream (Fig. 3, SEQ ID NO. 2) contains 75.6% A+T as compared to 63.5% A+T on average in the ϕLC3 genome (Lillehaug, et al., 1991). Flanking the core sequence several direct and inverted repeats, assymetrically located relative to the core, are seen, which also is typical for attachment site regions. These sequence repeats probably represent functional elements of the ϕLC3 attP site, possibly serving as recognition sites for proteins involved in the site-specific recombination. PCR amplification and DNA sequencing of the attR and attL junction regions in two lysogenic isolates of IMN-C17, five lysogenic isolates of IMN-C18, and øLC3 parental strain IMN-C3 demonstrated that the same attP and attB sites had been used for integration in all the lysogenic strains examined. This result supports the Southern analysis results (Fig. 1) and previous data (Lillehaug et al., 1991) indicating that the attB site described here could be the only attachment site or at least a strongly preferred site used for øLCR integration.

The øLC3 attB site is located within a protein coding region

The attB core sequence was found to be located

within an open reading frame 15 bp upstream from the stop codon of this reading frame (Fig. 5) (SEQ ID NOS 8 and 9). Thus, øLC3 integrates into this open reading frame but, interestingly, the integration leads to the substitution of the five C-terminal amino acids with five other residues encoded by phage DNA at the attL junction region (Fig. 5) (SEQ ID NOS 7 and 10). The size of this open reading frame is thus preserved in the lysogen, and it is possible that the slight change in the C terminus caused by the integration does not disrupt the function of the putative gene product. The actual size of this open reading frame has not yet been determined, but preliminary sequence analysis indicates a length of more than 600 bp. A search for amino acid sequence similarities did not reveal any significant similarity between this putative protein and other polypeptides in current data bases.

Identification of the ϕLC3 int gene and comparison to other site-specific recombinases

Sequence analysis of a 1.7 kb region surrounding the ϕLC3 attP site, between the HindIII and the HincII sites as shown in Fig. 1, revealed a 1.1 kp open reading frame ending 87 bp to the left of the core sequence (Fig. 3) (SEQ ID NO. 2). This reading frame, encoding a basic protein of 374 amino acids, begins with an ATG initiation codon preceded by an 11-bp putative ribosome-binding sequence with almost complete homology to the 3' end of the L. lactis 16S ribosomal RNA. Computer search in protein databases revealed extensive homology between the putative orf374 gene product and several phage and plasmid site-specific recombinases belonging to the integrase family (Argos et al., 1986; Poyart-Salmeron et al., 1989), strongly suggesting that orf374 represents the ϕLC3 site-specific recombinase. The location of attP close to the C-terminal end of the integrase gene is also a typical organization of these integrative systems.

The DNA sequence analysis revealed a cleavage site for restriction enzyme BlnI within orf374 (Fig. 3). This BlnI site was found to be unique in the ϕLC3 genome, thereby providing an easy strategy for in vitro construction of a mutation in orf374. A 4-bp insertion mutation was made by Klenow-fill in of BlnI-digested ϕLC3 phage DNA, blunt-end ligation and transfection of L. lactis subsp. cremoris strain IMN-C18. The construction was confirmed by sequencing through this region after amplification by PCR. The 4-bp insertions leads to replacement of the 34 C-terminal amino acid with a 22 amino acid chain resulting from the frameshift mutation. Thus, the mutated gene will encode a 362 amino acid fusion protein retaining the first 340 amino acids of the orf274 gene product. Mutant ϕLC3 carrying this 4-bp insertion was unable to give rise to stable lysogens although the plaques formed were turbid, and indistinguishable from ϕLC3 wild-type plaques. Plaques of wild-type ϕLC3 are turbid because lysogens, that have acquired immunity to ϕLC3, arise and grow during the formation of the plaque. When cells from plaques of the ϕLC3 mutant were picked and streaked onto agar plates, the colonies formed had an irregular appearance and were more transparent than colonies isolated from ϕLC3 wild-type plaques. Repeated attempts to isolate stable lysogenic clones from plaques of the mutant phage failed, and the turbidity in the plaques is probably due to growth of abortive lysogens in which ϕLC3 is not integrated and therefore unstable. Furthermore, the 4 bp insertion in orf374 was complemented by co-infection of the ϕLC3-C1 clear plaque mutant, confirming that the insertion mutation is affecting a diffusible factor which can be supported in trans, and not a structural element of the ϕLC3 attachment-site. Thus, in addition to the similarity between orf374 and other phage integrases, the ϕLC3 mutant carrying the 4-bp insertion in orf374 is phenotypically similar to phage λ int

mutants (Gottesman & Yarmolinsky, 1968). This identifies orf374 as the φLC3 int gene. The construction of a φLC3 mutant in which the C-terminal region of int and the complete attP site is deleted by removal of the 398-bp BlnI-XbaI fragment (Figs. 1 and 3), confirmed the abortive lysogen phenotype. We call the int insertion and the int-attP deletion mutants φLC3 intins 1 and φLC3 del2, respectively.

The φLC3 Int protein is a member of the integrase family of recombinases.

As mentioned above, amino acid sequence similarity between the φLC3 Int protein and several phage and plasmid integrases was found. The strongest overall homology was found to the integrase of temperate phage L54a, from Staphylococcus aureus, which shares 20.9% amino acid identity with the φLC3 integrase (Fig. 6A). Although the Int family of recombinases are a diverse group of enzymes, all members share three moderately conserved regions (Abremski, et al., 1992; Argos et al., 1986; Poyart-Salmeron et al., 1989), that are believed to make up the catalytic site (Abremski et al., 1992; Pargellis et al., 1988). The φLC3 integrase was also found to possess these conserved regions, including four invariant amino acid residues (Fig. 6B) (Abremski et al., 1992). Within the C-terminal conserved region, the strongest sequence similarity was found to transposase A (TnpA) from Staphylococcus aureus transposon Tn554 (Murphy et al., 1985), which shares 53.8% amino acid sequence identity with the øLC3 integrase. Furthermore, many of the sequence differences represent convervative amino acid substitutions. The øLC3 intins1 mutation disrupts the int reading frame 12 codons upstream of the codon for the invariant tyrosine residue, thus demonstrating that the region carrying the invariant tyrosine residue is also essential for the function of the øLC3 integrase. (Fig. 6B).

SUMMARY

We have demonstrated that the temperate lactococcal bacteriophage φLC3 integrates into the bacterial chromosome through site-specific recombination between phage and bacterial attachment sites, attP and attB. The attachment sites have been mapped and sequenced. A 9-bp core region, within which the DNA crossover takes place, has been identified. The integration requires a φLC3-encoded site-specific recombinase clearly related to the Int family of recombinases found in various plasmids, transposons, and temperate bacteriophages. The sequence similarity to other integrases, especially in the conserved C-terminal region, in addition to the presence of the four invariant amino acid residues, suggests that φLC3 uses the same mechanism for integration as the other integrative systems belonging to the Int family.

The location of the attP site immediately downstream from the int gene is a typical organization of this kind of integrative elements. Only in in temperate coliphage φ80 and P4, the attP site is located upstream from the int gene. The size of the common core sequence varies from a hexanucleotide sequence in Tn554 (Murphy & Lofdal, 1984) to a 182 bp region in temperate bacteriophage HP1 (Goodman & Scocca, 1989). The 9-bp φLC3 core is to our knowledge the smallest core sequence found in temperate bacteriophages. The exact boundaries of the functional φLC3 attP site are not yet known, but the sequence needed for integration is probably located within the 290-bp region between the end of the int gene and XbaI site (Fig. 3). The direct and inverted sequence repeats located in this region could represent recognition sites for the φLC3 integrase and other proteins binding within attP. The attP site of phage λ, which comprises about 240 bp, contains two types of binding sites for the λ integrase, the core type and the

arm type of Int binding sites, in addition to sites for binding of host integration factor (IHF), factor for inversion stimulation (FIS) and λ excisionase (Xis) (Thompson & Landy, 1989, Ball and Johnson, 1991a and b).

A lactococcal strain lysogenized with the int-defective mutant ϕLC3 intins1 by complementation with a ϕLC3 clear-plaque mutant, produces about 10^4-fold less spontaneously released phage than a strain lysogenic with ϕLC3 wild-type indicating that the ϕLC3 int gene is needed for efficient phage excision as well as for integration, as also demonstrated for other temperate phages.

EXAMPLE 2

MATERIALS AND METHODS

Bacterial strains, plasmids and phage

L. lactis subsp. lactis strain LM0230 was used in this study. Plasmid vector pG⁺host5 (Appligene, Illkirch Cedex, France) encodes erythromycin resistance and is a replication-thermosensitive derivative of the lactococcal plasmid pGK12 (Maguin et al., 1992). pINT1 is a pG⁺host5-derivative containing a 1.6-kb DNA fragment harboring the øLC3 int-attP region inserted into the polylinker between the BamHI and SalI restriction sites. Strain L. lactis subsp. lactis LMG2231 is a derivative of strain LM0230 carrying pINT1 in the integrated state (grown at 37°C). Strain LMG2230 is a LM0230-derivative carrying the non-replicative Em^r-int-attP cartridge inserted into attB. L. lactis was grown in M17 broth or on M17 agar (Terzaghi and Sandine, 1975). øLC3 phage and DNA were prepared as previously described (Lillehaug et al., 1991).

DNA amplification

Characterisation of the øLC3 integrase gene and

attP region is described in Example 1. A 1.6 kb fragment containing the attP site and the int gene, was amplified by polymerase chain reaction (PCR) (Saiki et al., 1988) using a GeneAmp Kit (Perkin-Elmer Cetus). The oligonucleotides used in the PCR reaction were the leftward primer, L (5'-ACTAGTCGACGGATGATTGTGGGATTTCATCC-3') and the rightward primer, R (5'-CGGGATCCTGTCTGACGGCTGGGTAATG-3'), containing restriction sites for SalI and BamHI (underlined), respectively. Annealing of the primers to the øLC3 DNA template was performed at 52°C. The L and R primers hybridize to a region 207 to 228 nucleotides to the right of the 9-bp core region of attP and to a region 154 to 173 nucleotides upstream from the ATG start codon of the int gene (Fig. 3), respectively. PCR amplification of attB, and of the attL and attR junctions, was performed using, respectively, set 5, 6, and 7 of the specific PCR primer-sets as described in Example 1. The oligonucleotides were synthesized at The Biotechnology Center of Oslo, University of Oslo, Norway.

General recombinant DNA methodology

Agarose gel electrophoresis and molecular cloning were performed by standard procedures (Sambrook et al., 1989) unless otherwise stated. Southern analysis was done by blotting onto a GeneScreen Plus membrane (New England Nuclear, Boston, Mass.) with an LKB vacuum blotter as recommended by the manufacturer. The DNA on the membrane was hybridized with probes that had been labeled with ^{32}P by the use of a Random Primed DNA Labeling Kit (Boehringer Mannheim) and [α-^{32}P]dATP (Amersham), or by PCR DNA probes that were labelled with [α-^{32}P]dATP (Amersham) during amplification). Hybridization was performed as described elsewhere (Church and Gilbert, 1984). Extraction of DNA from agarose gels was done by the use of a GeneClean Kit (Bio 101, Inc., La Jolla, Calif.). Plasmids and total DNA

from L. lactis were prepared essentially as described elsewhere (Leenhouts et al., 1989).

DNA sequence determination

Nucleotide sequence determination was performed by the dideoxy chain termination method (Sanger et al., 1977) with the SequenaseII DNA sequencing kit (United States Biochemical) and [α-^{35}S]ATP (Amersham). The DNA region upstream from the int gene was sequenced as double-stranded plasmid DNA (Perbal, 1988) using a cloned øLC3 restriction fragment. PCR-amplified fragments containing the attL and attR sites were generated using biotin-labelled primers and sequenced as single-stranded DNA prepared by magnetic separation with streptavidin-coated Dynabeads M-280 (Dynal As, Oslo, Norway) as recommended by the manufacturer.

Construction and chromosomal integration of an Em^r-int-attP cartridge

A 1.2-kb HindIII-ClaI Em^r-containing DNA restriction fragment from pVS2 was first subcloned into the polylinker of the cloning vector pBluescript SK+ (Stratagene, La Jolla, Calif.), between the HindIII and ClaI sites. This was done in order to place the Em^r gene within a polylinker to facilitate further transfer to other constructs. The Em^r-containing derivative of pBluescript SK+ was digested with BamHI and SalI to give a Em^r-containing fragment flanked by BamHI and SalI restriction sites. This 1.2-kb fragment was ligated together with a 1.6-kb int-attP PCR fragment which had been digested with BamHI and SalI and purified by agarose gel electrophoresis, to create a 2.8-kb circular construct containing Em^r, int and attP. The ligation mixture was used for electrotransformation of L. lactis subsp. lactis strain LM0230 that had been made competent by growth in the presence of 1.5% of glycine in an osmotically stabilized medium (Holo and Nes, 1989).

Transformants were selected by plating onto M17 plates supplemented by 0.25 M Na-succinate, 0.5% glucose, 10 mM $MgCl_2$, 10 mM $CaCl_2$, and 1 μg of erythromycin per ml.

Construction of a site-specific integrating plasmid

The 1.6-kb int-attP-containing DNA fragment described above was ligated between the BamHI and SalI restriction sites of pG$^+$host5 and transformed into L. lactis subsp. lactis strain LM0230 by electroporation. Selection of transformants was done on M17 plates as above at 30°C. For curing of the plasmid and selection of integrants transformants were grown at 37°C.

Efficiency of Int-attP-mediated plasmid integration

The frequency by which pINT1 integrates into the lactococcal chromosome was measured essentially as described (Biswas et al., 1993). Strain LM0230-pINT1 grown overnight at 30°C in M17 broth containing 1% glucose and 1 μg of erythromycin per ml was diluted 100-fold in the same medium and further grown at 30°C for 2 hours. The culture was then shifted to 37.5°C for 3 hours, and samples were diluted and plated at 37.5°C on plates containing 1 μg of erythromycin per ml for detection of integration events and at 30°C to determine the viable cell count. The frequency of integration was estimated as the ratio of the number of Em^r CFU at 37.5°C to the total CFU at 30°C under non-selective conditions.

Stability of integrants

The stability of the Em^r phenotype of the integrants was examined by growing the strains for 100 generations under non-selective conditions in M17 broth supplemented by 1% glucose, by transferring 100 colonies of each strain with toothpicks from nonselective plates onto selective and nonselective plates. Strain LMG2230 was grown at 30°C, whereas strain LMG2231 was grown at 37°C.

RESULTS

Chromosomal integration of a non-replicative cartridge

A PCR-generated DNA fragment harboring the øLC3 int gene and attP site was ligated to a fragment containing the Em^r gene, to generate a circular non-replicative Em^r-int-attP-containing molecule, and used to transform L. lactis subsp. lactis strain LM0230. One µg of the ligation mixture gave 18 Em^r transformants. Analysis by PCR and Southern hybridization of chromosomal DNA extracted from 11 of these transformants showed that the Em^{r}int-attP cartridge had integrated in the lactococcal chromosome. One such integrant, termed LMG2230, was used for further analysis, and was found to carry the cartridge in an integrated manner as illustrated in Fig. 8B. Amplification of LMG2230 DNA using primers which specifically amplifies the attB site or the host-phage junctions (attL and attR) showed that attB is disrupted while junction sites corresponding to the attL and attR sites in a normal øLC3 lysogen has been formed (Fig. 10, lanes 2, 3, and 4). The attB-specific primers resulted in a PCR-product of about 3 kb (lane 2), corresponding in size to of the complete Em^r-int-attP cartridge plus bacterial DNA localized between the cartridge and the PCR primers (ca. 2800 + 202bp). The attL and attR specific primers, which are designed to hybridize to prophage and bacterial DNA, yielded PCR products of 327 and 282 bp, respectively. Sequence analysis of the amplified junction fragments showed that the nucleotide sequence was identical to those from a lysogen, which is characterized in Example 1, demonstrating that integration has been performed through site-specific recombination between attB and attP exactly as during lysogenization of L. lactis by øLC3. The use of Em^r-int-attP-specific DNA probes in Southern analysis (Fig. 9, lanes 1 and 2) confirmed that a single copy of the Em^r-int-attP cartridge has been inserted into the attB site

in an orientation-specific manner as schematically illustrated in Fig. 8B.

Construction of an int-attP-containing integrative plasmid

Thermosensitive plasmid vectors in which replication can be blocked by increasing the temperature from 30°C to 37°C have recently been developed, and have been successfully used as gene inactivation and replacement systems for Gram-positive bacteria (Biswas et al., 1993; Maguin et al., 1992). We cloned the int-attP-containing fragment into such a vector (pG$^+$host5), and selected for integrants by temperature shift-up. At 37°C only integrants carrying the plasmid in an integrated form can grow in the presence of erythromycin. The int-attP-containing pG$^+$host5 derivative, termed pINT1 (Fig. 8A), integrated with high efficiency into the L. lactis chromosome. Following temperature shift-up, ca. 20% of the cells retained the Em^r phenotype (Table 2). As a control, the vector plasmid, pG$^+$host5, resulted in a low frequency (ca. 1 x 10^{-5}) of Em^r colonies after a shift to 37°C. A low background of nonspecific integration of pG$^+$host5 has been observed earlier (Biswas et al., 1993). One of the pINT1 integrants, termed LMG2231, was used for further analysis. As for the nonreplicating cartridge described above, PCR analysis of LMG2231 DNA showed that attB is disrupted while attL and attR junction sites have been generated (Fig. 10, lanes 5, 6 and 7). Different from LMG2230, the attB-specific primers did not yield any visible PCR product, which is probably due to the large size of the integrated plasmid (6.8 kb). Analysis of LMG2231 DNA by Southern hybridization (Fig. 9, lanes 3 and 4) were consistent with the results of the PCR-analysis, and confirmed that a single copy of pINT1 had integrated into attB according to the schematic illustration in Fig. 8A.

TABLE 2 - Efficiency of integration[a]

Plasmid	CFU under nonselective conditions (30°C)	CFU under selective conditions (EM^r, 37°C)	Integration frequency
pINT1	~2.7×10^8	~5.1×10^7	~0.2
pG^+host 5	~4.5×10^8	~5.1×10^7	~1×10^{-5}

[a] Each result is an average of three independent experiments.

Stability of the Em^r phenotype of the integrants

Strain LMG2230 and LMG2231 were grown in M17 broth for 100 generations under nonselective conditions and then tested for the presence of the Em^r gene. Of 100 colonies from each strain, all retained the Ems phenotype, demonstrating that both integrative constructs give rise to integrants that are genetically quite stable and that these systems are well suited for stable insertion of foreign DNA into the L. lactis chromosome.

Expression of the øLC3 int gene

Upstream from the int structural gene a nearly consensus promoter sequence was revealed (Fig. 3) (SEQ ID NO. 2). The region complementary to the oligonucleotide primer, R, used for amplification of the int gene was chosen to include the putative int promoter in the amplified DNA fragment (Fig. 3) (SEQ ID NO. 2). Expression of the int function from the cloned PCR product indicates that the proposed putative promoter could be the promoter from which the øLC3 int gene is normally expressed. An 8-bases inverted repeat sequence overlapping the putative -35 promoter sequence (Fig. 3) (SEQ ID NO. 2) could possibly represent a regulatory

protein-binding element involved in the regulation of int expression.

SUMMARY

The results above demonstrate that the øLC3 integration system can function as an efficient tool for integration of DNA into the lactococcal chromosome. The advantage of using the øLC3-based non-replicative integrating cartridge is that it only contains sequences derived from a phage normally present in lactococcal starter cultures, that it mediates integration into a site that normally functions as an insertion site, and that the use of heterologous plasmid DNA can be avoided. The precise integration and high integration frequency of pINT1 (~0.2) probably makes this plasmid the most efficient system for single-copy chromosomal integration of foreign DNA into lactococci.

LITERATURE CITED

Abremski, K.E. and R.H. Hoess. 1992. Evidence for a second conserved arginine residue in the integrase family of recombination proteins. Protein Eng. 5: 87-91.

Altschul, S.F., W. Gish, W. Miller, E.W. Myers and D.J. Lipman. 1990. J. Miol. Biol. 215: 403-410.

Argos, P.A. Land, K. Abremski, J.B. Egan, E. Haggård-Ljungquist, R.H. Hoess, M.L. Kahn, B. Kalionis, S.V.L. Narayana, L.S. Pierson, N. Sternberg and J.N. Leong. 1986. The integrase family of site-specific recombinases: regional similarities and global diversity. EMBO J. 5: 433-440.

Ball, C.A., and R.C. Johnsen. 1991a. Efficient excission of phage lambda from the Escherichia coli chromosome requires the Fis protein. J. Bacteriol. 173: 4027-4031.

Ball, C.A., and R.C. Johnsen. 1991b. Multiple effects of Fis on integration and the control of lysogeny in phage lambda. J. Bacteriol. 173: 4032-4038.

Biswas, I., A. Gruss, S.D. Ehrlich, and E. Maguin. 1993. High-efficiency gene inactivation and replacement system for Gram-positive bacteria. J.Bacteriol. 175: 3628-3635.

Church, G.M., and W. Gilbert. 1984.. Genomic sequencing. Proc. Natl. Acad. Sci. USA. 81: 1991-1995.

Davidson, B.E., I.B. Powell, and A.J. Hillier, 1990. Temperate Bacteriophages and lysogeny in lactic acid bacteria, FEMS Microbiol. Rev. 87: 79-90.

Goodman, S.D., and J.J. Scocca. 1989. Nucleotide sequence and expression of the gene for the site-specific integration protein from bacteriophage HP1 of Haemophilus influenzae. J. Bacteriol. 171: 4232-4240.

Gottesman, M.E., and M.B. Yarmolinsky. 1968. Integration-negative mutants of bacteriophage Lambda. J. Mol. Biol. 31: 487-505.

Guchte, Van de M., J. Kok and G. Venema. 1992. Gene expression in Lactococcus Lactis. FEMS Microbiol. Rev. 88: 73-92.

Hall, L.M., and G. Vockler. 1987. The region of the IncN plasmid R46 coding for resistance to beta-lactam antibiotics, streptomycin/spectinomycin and sulphonamides is closely related to antibiotic resistance segments found in IncW plasmids and in Tn21-like transposons. Nucleic. acids Res. 15: 7491-7501.

Hanahan, D. 1983. Studies on transformation of Escherichia coli with plasmids. J. Mol. Biol. 166: 557-580.

Hartley, J.L. and J.E. Donelson. 1980. Nucleotide sequence of the yeast plasmid. Nature (London) 286: 860-864.

Higgins, D.G. A.J. Pleasby and R. Fuchs. 1992. CLUSTAL V: improved software for multiple sequence alignment. Comput. Appl. Biosci. 8: 189-191.

Holo, H., and I.F. Nes. 1989. High-frequency transformation, by electroporation of lactococcus lactis subsp. cremoris grown with glycine in osmotically stabilised media. Appl. Environ. Microbiol. 55: 3119-3123.

Horng, J.S. et al., 1991. J. Bacteriol. 173: 7573-7581.

Klemm, P. 1986. Two regulatory fim genes, fimB and fimE, control the phase variation of type 1 fimbriae in Escherichia coli. EMBO J. 8: 1389-1393.

Kubo, A.A. Kusukawa and T. Komano. 1988. Nucleotide sequence of the rci gene encoding shufflon-specific DNA recombinase in tghe IncI1 plasmid R64: Homology to the site-specific recombinases of integrase family. Mol. Gen. Genet. 213: 30-35.

Lakshmidevi, G. et al., 1989. Circular Permutation of the Genome of a temperate bacteriophage from Streptococcus Cremoris BK5. Appl. Env. Microbiol. 54: 1039-1045.

Lee, M.H., L. Pascopella, W.R. Jacobs Jr., and G.F. Hatfull. 1991. Site-specific integration of mycobacteriophage L5: Integration-proficient vectors for Mycobacterium smegmatis, Mycobacterium tuberculosis, and bacille Calmette-Guérin. Proc. Natl. Acad. Sci. USA 88: 3111-3115.

Leenhouts, K., J. Kok, and G. Venema. 1989 Campbell-like integration of heterologous plasmid DNA into the chromosome of Lactococcus lactis subsp. lactis. Appl. Environ. Microbiol. 55: 394-400.

Lillehaug, D., B.H. Lindqvist, and N.K. Birkeland. 1991. Characterisation of ϕLC3, a Lactococcus lactis subsp. cremoris temperate bacteriophage with cohesive single-stranded DNA ends. Appl. Environ. Microbiol. 57:3206-3211.

Leong, J.M., S.E. Nunes-Düby, A.B. Oser, C.F. Lesser, P. Youderian, M.M.Susskind, and A. Landy. 1986.

Structural and regulatory divergence among site-specific recombination genes of lambdoid phage. J. Mol. Biol. 189: 603-613.

Macrina, F.L., R.P. Jones, J.A. Tobian, D.L. Hartley, D.B. Clewell and K.R. Jones. 1983. Novel shuttle plasmid vehicles for Escherichia-Streptococcus transgeneric cloning. Gene 25: 145-150.

Maguin E. et al., J. Bacteriol. 1992. 174: 5633-5638.

Mahillon, J., and D. Lereclus. 1988. Structural and functional analysis of Tn4430: identification of an integrase-like protein involved in the co-integrate-resolution process. EMBO J. 7: 1515-1526.

Murphy, E., Huwyler, and de Freire Bastos M. d-C. 1985. Transposon Tn554: Complete nucleotide sequence and isolation of transposition-defective and antibiotic-sensitive mutants. EMBO J. 4: 3357-3365.

Murphy, E., and S. Löfdahl. 1984. Transposition of Tn554 does not generate a target duplication. Nature (London) 307: 292-294.

Norrander, J., T. Kempe, and J. Messing. 1983. Construction of improved M13 vectors using oligonucleotide-directed mutagenesis. Gene 26: 101-106.

Ouelette, M., and P. Roy. 1987. Nucleic Acids Res. 15: 1005.

Pargellis, C.A., S.E. Nunes-Düby, L. Moitoso de Vargas and A. Landy. 1988. Suicide recombination substrates yield covalent λ integrase-DNA complexes and lead to the identification of the active site tyrosine. J. Biol. Chem. 263: 7678-7685.

Parker, J.D., P.S. Rabinovitch, and G.C. Burmer. 1991. Targetted gene walking polymerase chain reaction. Nucleic Acids Res. **19**: 3055-3060.

Perbal, B. 1988. A practical guide to molecular cloning, 2nd ed. John Wiley & Sons, Inc., N.Y.

Pearson, W.R., and D.J. Lipman. 1988. Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. USA 85: 2444-2448.

Polzin & McKay, Appl. Env. Microbiol. 1992. **58**: 476-484.

Poyart-Salmerson, C.P. Trieu-Cuot, C. Carlier, and P. Courvalin. 1989. Molecular characterisation of two proteins involved in the excision of the conjugative transposon Tn1545: homologies with other site-specific recombinases. EMBO J. **8**: 2425-2433.

Prasad et al., 1987.

Raya R.R., C. Fremaux, G.D. De Antoni and T.R. Klaenhammer. 1992. Site specific integration of temperate bacteriophage ϕadh into the Lactobacillus gasseri chromosome and molecular characterisation of the phage (attP) and bacterial (attB) attachment sites. J. Bacteriol. **174**(17): 5584-5592.

Romero, D.A., and T.R. Klaenhammer. 1992. 15946-medicated integration of heterologous DNA into the genome of Lactococcus lactis subsp. lactis. Appl. Environ. Microbiol. **58**: 699-702.

Saiki, R.K., D.H. Gelfand, S. Stoffel, S. Scharf, H. Russel, G.T. Horn, K.B. Mullis, and H. Erlich. 1988. Primer-directed enzymatic amplification of DNA with a

thermostable DNA polymerase. Science 239: 487-491.

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2th ed., Vol. 1-3. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Sander, M.E., and J.W. Schultz. 1990. Cloning of phage resistance genes from Lactococcus Lactis subsp. cremoris KH. J. Dairy Sci. 73: 2044-2053.

Sanger, F., S. Nicklen and A.R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Nat. Acad. Sci. USA, 74: 5463-5467.

Scheirlinck, T., J. Mahillon, H. Joos, P. Dhaese, and F. Michiels. 1989. Integration and expression of α-amylase and endogluconase genes in the Lactobacillus plantarium chromosome. Appl. Environ. Microbiol. 55: 2130-2317.

Terzaghi, B.E., and W.E. Sandine. 1975. Improved medium for lactic streptococci and their bacteriophages. Appl. Microbiol. 29: 807-813.

Thompson, J.F., and A. Landy. 1989. Regulation of bacteriophage lambda site-specific recombination, p. 1-22. In D.E. Berg and M.M Howe (ed.), Mobile DNA. American Society for Microbiology, Washington, DC.

Yanisch-Perron, C., J. Veira and J. Messing. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33: 103-119.

Ye, Z.-H., Buranen, S.L. and C.Y. Lee. 1990. Sequence analysis and comparison of int and xis genes from staphylococcal bacteriophage L54a and ϕ11. J.

Bacteriol. 172: 2568-2575.

Ye, Z.H., and C.Y. Lee. 1989. Nucleotide sequence and genetic characterisation of staphylococcal phage L54a int and xis genes. J. Bacteriol. 171: 4146-4153.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

(A) NAME: Norwegian Dairies Association
(B) STREET: Breigata 10
(C) CITY: Oslo
(E) COUNTRY: Norway
(F) POSTAL CODE (ZIP): N-0187

(A) NAME: LILLEHAUG, Dag
(B) STREET: Laboratory of Microbial Gene Technology, P.O. Box 5051
(C) CITY: Oslo
(E) COUNTRY: Norway
(F) POSTAL CODE (ZIP): N-1432

(A) NAME: BIRKELAND, Nils-kare
(B) STREET: Laboratory of Microbial Gene Technology, P.O. Box 5051
(C) CITY: Oslo
(E) COUNTRY: Norway
(F) POSTAL CODE (ZIP): N-1432

(A) NAME: DZIEGLEWSKA, Hanna Eva
(B) STREET: Frank B. Dehn & Co., Imperial House, 15-19 Kingsway
(C) CITY: London

(E) COUNTRY: United Kingdom
(F) POSTAL CODE (ZIP): WC2B 6UZ

(ii) TITLE OF INVENTION: Bacteriophage phi-LC3 based vector system for transformation of bacteria

(iii) NUMBER OF SEQUENCES: 10

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(v) CURRENT APPLICATION DATA:
APPLICATION NUMBER: WO PCT/GB94/
(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: GB 9303850.3
(B) FILING DATE: 25-FEB-1993

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

TTCTTCATG 9

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1833 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

TGTCTGACGG CTGGGTAATG TATTTAGATT TCAATAATGG TGTAGTTAAA TAAACAAAAA 60

AATCCGCTCA AGTTTGACGA CAAGGGGCGGATTTAAACTA TAAAATAGTA TAAAGGCTTT 120

TAAGAAGCTT TTTACTATAC CATTTTATCA GAAATGAGGT ATAAAAAGCA AATATGGCTA 180

CATATCAAAA GCGTGGTAAA ACTTGGCAGT ATTCAATATC AAGAACAAAA CAAGGACTTC 240

CTCGTCTAAC AAAGGGTGGT TTTTCTACAA AGTCCGATGC ACAAGCTGAA GCAATGGATA 300

TTGAAAGCAA ACTAAAAAAA GGATTTATTG TTGACCCCAT TAAGCAAGAA ATTTCCGAAT 360

ATTTTAAAGA CTGGATGGAA CTTTATACGA AAAATGCAAT TGATGAAATG ACTTATAAAG 420

GTTATGAGCA AACGTTAAAA TATTTAAAAA CCTATATGCC AAATGTTTTA ATTTCCGAAA 480

TAACAGCATC TTCTTATCAA AGAGCGCTAA ATAAATTTGC TGAAACACAC GCCAAAGCAT 540

CTACAAAAGG GTTTCATACT AGAGTTAGAG CATCTATTCA ACCACTCATT GAAGAGGGAC 600

GACTGCAAAA AGATTTTACC ACTCGTGCAG TAGTTAAAGG TAATGGAAAT GATAAAGCCG 660

AGCAAGACAA GTTTGTAAAT TTTGATGAAT ACAAGCAATT AGTTGATTAT TTCAGAAATA 720

GACTTAATCC AAACTATTCA TCTCCCACTA TGCTGTTTAT AATTTCAATT ACTGGCATGA 780

GAGCCAGTGA AGCTTTTGGC TTAGTCTGGG ATGATATTGA TTTTAATAAT AACACTATCA 840

AGTGTCGCAG AACTTGGAAT TACAGAAATA AAGTAGGTGG TTTCAAAAAG CCCAAAACAG 900

ATGCTGGAAT AAGAGATATT GTTATAGATG ATGAAAGTAT GCAATTGCTA AAAGATTTTA 960

GAGAACAGCA AAAAACATTA TTTGAAAGTT TGGGTATAAA ACCGATACAT GACTTTGTTT 1020

GTTATCATCC TTATAGAAAA ATAATAACTC TCTCAGCTTT GCAAAATACA TTAGAACATG 1080

CATTAAAAAA ACTAAAGATT TCTACTCCAC TTACTGTACA CGGTTTAAGG CATACTCATG 1140

CTTCTGTTCT CCTCTATCAT GGAGTTGATA TCATGACTGT TTCAAAACGC CTAGGACACG 1200

CAAGTGTGGC TATCACACAG CAAACCTATA TTCATATTAT AAAAGAGCTA GAAAATAAAG 1260

ATAAGGATAA AATAATTGAG CTGCTACTGG AGTTATAATT TTCTTACAAC AAAAATACAA 1320

CAAATCATTA AAAACTAAGA AGAAACCCAT TATCTAAAGC ATATATAACC TTTACTATGT 1380

AGTTCTTCAT GAACGATAAT AAACAATAAT AGAATAAATA TAAAATAAAA ACGGCTTAAT 1440

ATAGCCGTTT TTCCTGTTTT TAAAACAACT AAAAAATATA GAAATAAAAA ACTCGTACAA 1500

CAAAATTACA ACATATCATT ATGTTTCTGA TAGTATTGTT GTAAATTTAC AACAAACAAA 1560

AAAAGCCACT CCGAAGAATG GCTTAACTCT AGAAATAGGA TGAAATCCCA CAATCATCCC 1620

GAATATATTA TAGCACAAAA AAAGCGCCC CAGTTAGGAG AGGGACGCTTAGGATAAACTT 1680

ATGAAAAAAG TTTTTCGGAA TAAGAACATT ATATAACTTT CCGTTCTTTT TGTAAAGAAA 1740

AAGCCCCGGA GGGCGAGTGA TATATTAACC ATATATCAGC AAAAGATATT ACAAAAGTGC 1800

TTTTAAAACA CATGTTTTTT TGGTTGAACG GTC 1833

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 720 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

TTGCCACAAT ACTATCGACT TTTCAAAAGT CTAGTCGAAG AGAATGTGAC ACGCACAAAT 60

TCACAACTTA TTTCTGAAAA AATTGGGGTA GATGCTGCCA CAATTCGTCG TGATTTTTCT 120

CTCTTTGGGG AATTAGGACG ACGAGGCTAC GGATATGAAA CAAAAGTTCT TCGTGATTTC 180

TTTGGAGAAC TCCTTGGACA AGATCAAGAA ACTCATATCG CATTGATTGG AGTCGGAAAC 240

CTTGGACGTG CGCTTCTTCA TTATCAATTT CAAGACAGAA ATAAAATGAG AATTACTCAG 300

GCCTACGATA TTTCTGGAAA TCCACTAGTT GGAACTCAAA CAGATGATGG CATTCCAATT 360

TATAATATTT CTGATTTAGA GAAGAATGTT AAGAAATCAG ACATAAAAAC AGCCATTCTA 420

TCTGTTCGTA AGGAAAACGC TCAGGAAGTG GTTGATAGGG TTGTTAAAGC AGGAATCAAA 480

GGCTTTCTTA ACTTTGCACC TATTCGCTTG AAAGTCCCTT CAGATGTTGT TGTTCAATCT 540

ATTGATTTAA CTAAAGAATT GCAAACCTTG TTATTCTTCA TGGGAGCTCA AGAAGAATAA 600

AAGACAAGCA AAATTTTATC AAAACTATTA ATGATACCTA GGCCTTCGTG TGCTTAGGTA 660

TTTTATTTTC AAGCCATCAT CATTTTAATT TTTTCACAAA TTTTTAGTTT ATTATTTAGT 720

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 374 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Met Ala Thr Tyr Gln Lys Arg Gly Lys Thr Trp Gln Tyr Ser Ile Ser
1 5 10 15

Arg Thr Lys Gln Gly Leu Pro Arg Leu Thr Lys Gly Gly Phe Ser Thr
20 25 30

Lys Ser Asp Ala Gln Ala Glu Ala Met Asp Ile Glu Ser Lys Leu Lys
35 40 45

Lys Gly Phe Ile Val Asp Pro Ile Lys Gln Glu Ile Ser Glu Tyr Phe
50 55 60

Lys Asp Trp Met Glu Leu Tyr Thr Lys Asn Ala Ile Asp Glu Met Thr
65 70 75 80

Tyr Lys Gly Tyr Glu Gln Thr Leu Lys Tyr Leu Lys Thr Tyr Met Pro
85 90 95

Asn Val Leu Ile Ser Glu Ile Thr Ala Ser Ser Tyr Gln Arg Ala Leu
100 105 110

Asn Lys Phe Ala Glu Thr His Ala Lys Ala Ser Thr Lys Gly Phe His
115 120 125

Thr Arg Val Arg Ala Ser Ile Gln Pro Leu Ile Glu Glu Gly Arg Leu
130 135 140

Gln Lys Asp Phe Thr Thr Arg Ala Val Val Lys Gly Asn Gly Asn Asp
145 150 155 160

Lys Ala Glu Gln Asp Lys Phe Val Asn Phe Asp Glu Tyr Lys Gln Leu
165 170 175

Val Asp Tyr Phe Arg Asn Arg Leu Asn Pro Asn Tyr Ser Ser Pro Thr
180 185 190

Met Leu Phe Ile Ile Ser Ile Thr Gly Met Arg Ala Ser Glu Ala Phe
195 200 205

Gly Leu Val Trp Asp Asp Ile Asp Phe Asn Asn Asn Thr Ile Lys Cys
210 215 220

Arg Arg Thr Trp Asn Tyr Arg Asn Lys Val Gly Gly Phe Lys Lys Pro
225 230 235 240

Lys Thr Asp Ala Gly Ile Arg Asp Ile Val Ile Asp Asp Glu Ser Met
245 250 255

Gln Leu Leu Lys Asp Phe Arg Glu Gln Gln Lys Thr Leu Phe Glu Ser
260 265 270

Leu Gly Ile Lys Pro Ile His Asp Phe Val Cys Tyr His Pro Tyr Arg
275 280 285

Lys Ile Ile Thr Leu Ser Ala Leu Gln Asn Thr Leu Glu His Ala Leu Lys
290 295 300

Lys Leu Lys Ile Ser Thr Pro Leu Thr Val His Gly Leu Arg His
305 310 315 320

Thr His Ala Ser Val Leu Leu Tyr His Gly Val Asp Ile Met Thr Val
325 330 335

Ser Lys Arg Leu Gly His Ala Ser Val Ala Ile Thr Gln Gln Thr Tyr
340 345 350

Ile His Ile Ile Lys Glu Leu Glu Asn Lys Asp Lys Asp Lys Ile Ile
355 360 365

Glu Leu Leu Leu Glu Leu
370

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 49 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

ATATAACCTT TACTATGTAG TTCTTCATGA ACGATAATAA ACAATAATA 49

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 49 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

ATATAACCTT TACTATGTAG TTCTTCATGG GAGCTCAAGA AGAATAAAA 49

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 49 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

AAGAATTGCA AACCTTGTTA TTCTTCATGA ACGATAATAA ACAATAATA 49

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 49 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

AAGAATTGCA AACCTTGTTA TTCTTCATGG GAGCTCAAGA AGAATAAAA 49

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

Glu Leu Gln Thr Leu Leu Phe Phe Met Asn Asp Asn Lys Gln
1 5 10

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 49 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

AAGAATTGCA AACCTTGTTA TTCTTCATGG GAGCTCAAGA AGAATAAAA 49

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

Glu Leu Gln Thr Leu Leu Phe Phe Met Asn Asp Asn Lys Gln
1 5 10

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

Glu Leu Gln Thr Leu Leu Phe Phe Met Gly Ala Gln Glu Glu
1 5 10

Claims

1. A DNA molecule comprising the nucleotide sequence TTCTTCATG (SEQ ID NO. 1) serving as the core region of attP of phage øLC3 or of the corresponding attB site of a lactic acid bacterium host.

2. A DNA molecule as claimed in claim 1, comprising the nucleotide sequence TTCTTCATG (SEQ ID NO. 1) flanked by at least one additional nucleotide sequence such that said molecule is capable of serving either as the attP site of phage øLC3 or as the corresponding attB of the host.

3. A DNA molecule comprising a nucleotide sequence which acts as an integrase promoter and/or encodes an integrase and/or attP of phage øLC3, or a fragment thereof, substantially corresponding to all or a portion of the nucleotide sequence shown in Figure 3 (SEQ ID NO. 2) or a functionally equivalent sequence which is degenerate or substantially homologous with, or which hybridises with any such sequence.

4. A DNA molecule as claimed in claim 3, comprising nucleotides 1 to 173 containing the int promoter sequence or a functionally equivalent sequence and/or nucleotides 174 to 1298 encoding int or a functionally equivalent sequence, and/or the attP containing sequence comprising nucleotides 1299 to 1593, or a functionally equivalent sequence.

5. A DNA molecule as claimed in claim 3 or claim 4, comprising as the core region of attP, nucleotides 1383 to 1391 of Figure 3 (SEQ ID NO. 2).

6. A DNA molecule as claimed in claim 1 or claim 2, capable of serving as the attB site of the host,

comprising all or a portion of the nucleotide sequence shown in Figure 7 (SEQ ID NO. 3), or a functionally equivalent sequence which is degenerate or substantially homologous with, or which hybridises with any such sequence.

7. A vector system for introduction of foreign DNA into a bacterial host by site-spectic integration, characterised by utilization of phage øLC3 integration functions.

8. A vector system as claimed in claim 7, in the form of a single vector comprising a DNA molecule as claimed in any one of claims 1 to 5, together with a site for insertion of said foreign DNA.

9. A vector as claimed in claim 8, further comprising an expression control sequence for operative linkage to said foreign DNA.

10. A vector as claimed in claim 8 or 9, in the form of a phage øLC3 modified to introduce a site for insertion of said foreign DNA.

11. A vector as claimed in claim 8 or claim 9, comprising int and attP sequences as defined in claims 3 to 5.

12. A vector as claimed in any one of claims 8, 9 or 11, in the form of a non-replicatible module containing the said int and attP sequences.

13. A vector as claimed in any one of claims 8 or 9 or 11, in the form of a plasmid.

14. A vector as claimed in claim 13, wherein said plasmid further comprises one or more additional

elements selected from an origin of replication, a promoter for the integrase and one or more selectable markers.

15. A vector as claimed in claim 14, wherein said promoter comprises the promoter sequence at nucleotides 1 to 173 of Figure 3 (SEQ ID NO. 2).

16. A vector system as claimed in claim 7, wherein int and attP sequences as defined in claims 3 to 5, are carried on two or more separate vectors.

17. A vector system as claimed in claim 16, comprising a first vector carrying an integrase encoding sequence as defined in claim 3 or claim 4, and a second vector carrying an attP-containing sequence as defined in claims 3 to 5, together with a site for insertion of said foreign DNA.

18. A method for the introduction of foreign DNA into a bacterial host, said method comprising transforming said bacterial host with one or more vectors comprising a DNA molecule as claimed in any one of claims 1 to 5, together with said foreign DNA.

19. A method as claimed in claim 18, wherein said bacterial host is a lactic acid bacterium.

20. A method as claimed in claim 19, wherein said bacterial host is a lactococcus.

21. A method as claimed in claim 18 or claim 19, wherein said bacterial host has been modified to introduce an attB site as defined in any one of claims 1 to 3 or 6.

22. Bacterial host cells transformed according to any one of claims 18 to 21.

23. Use of phage øLC3 as a vector for introduction of foreign DNA into a bacterial host.

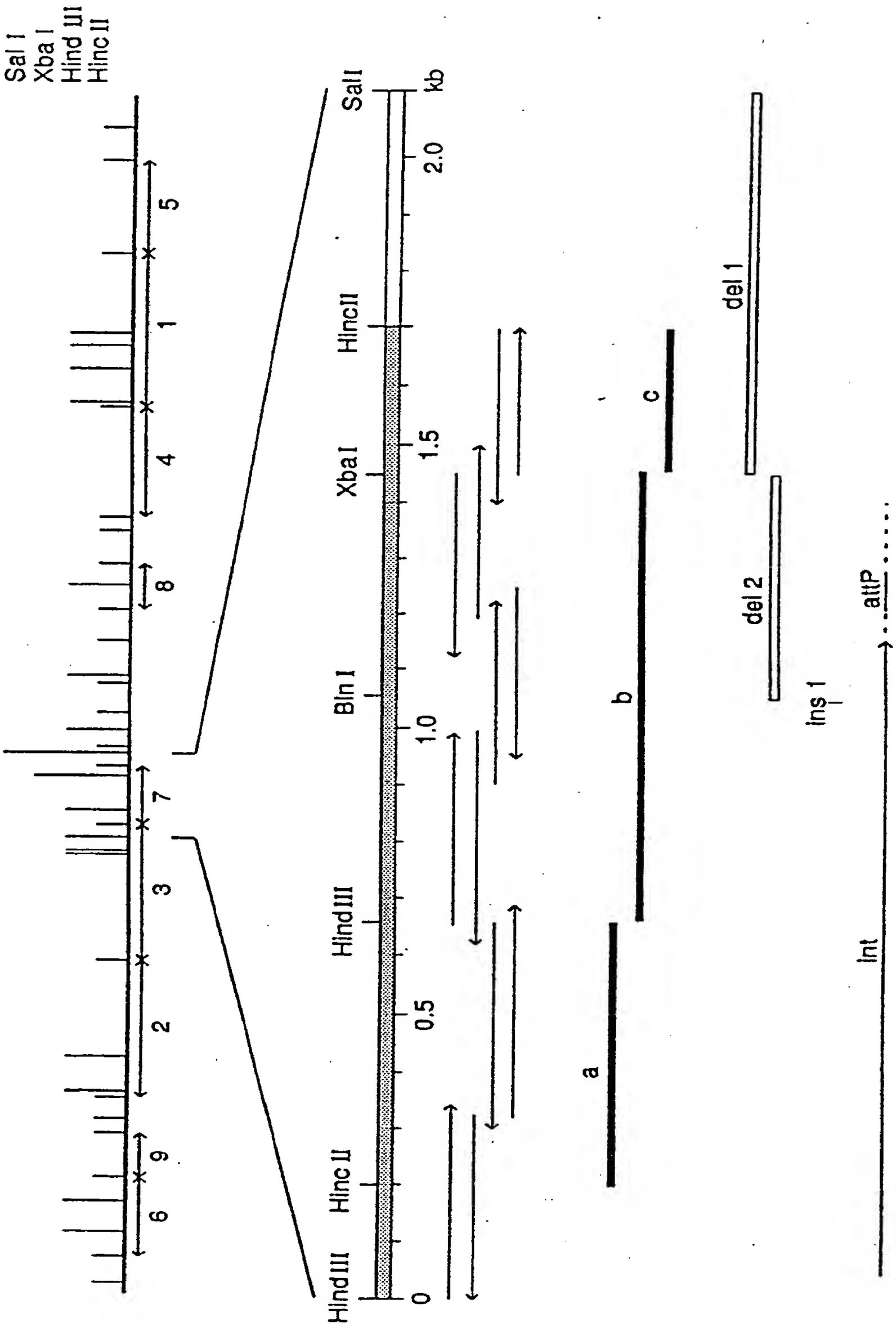

Figure 1

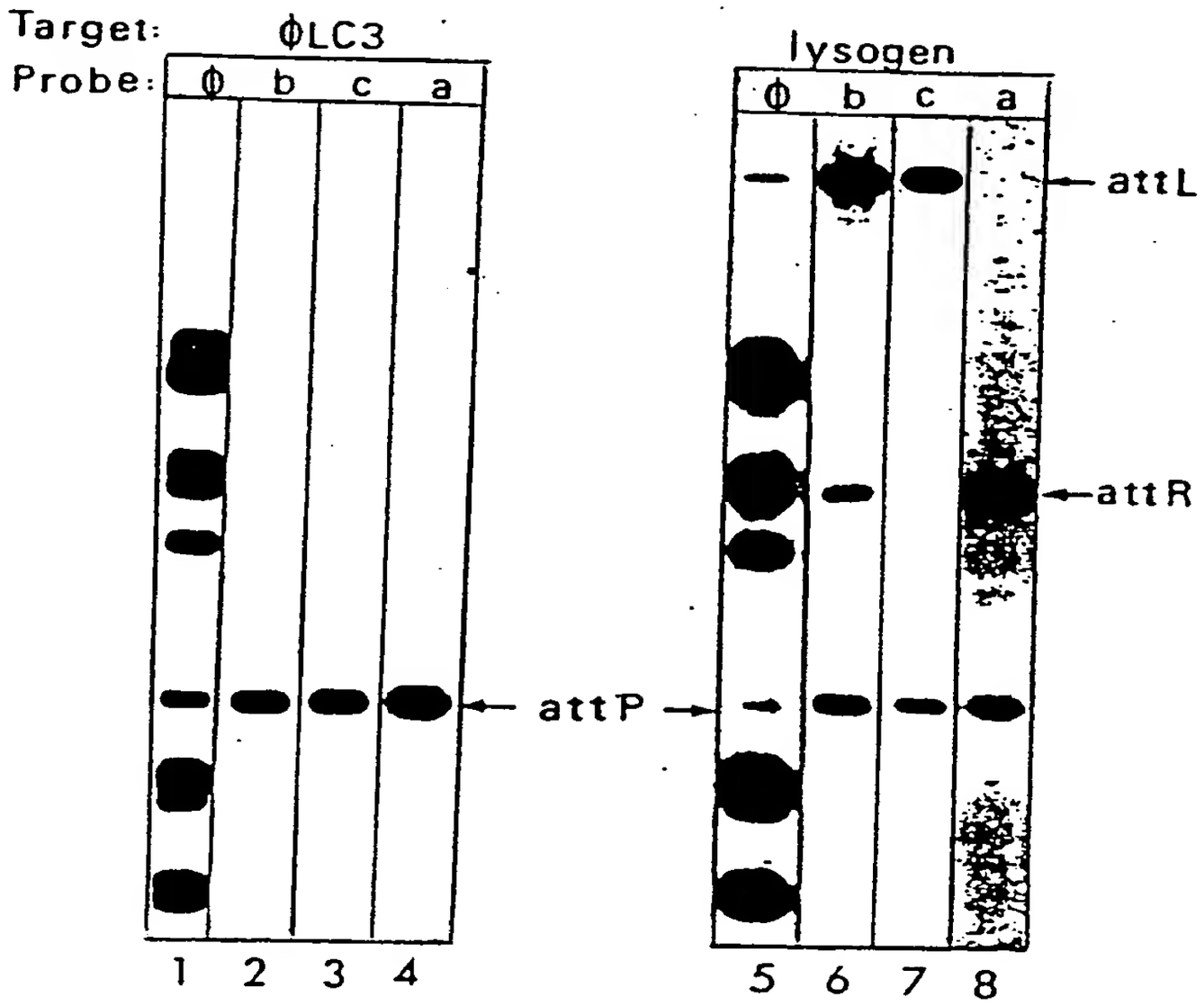

Figure 2

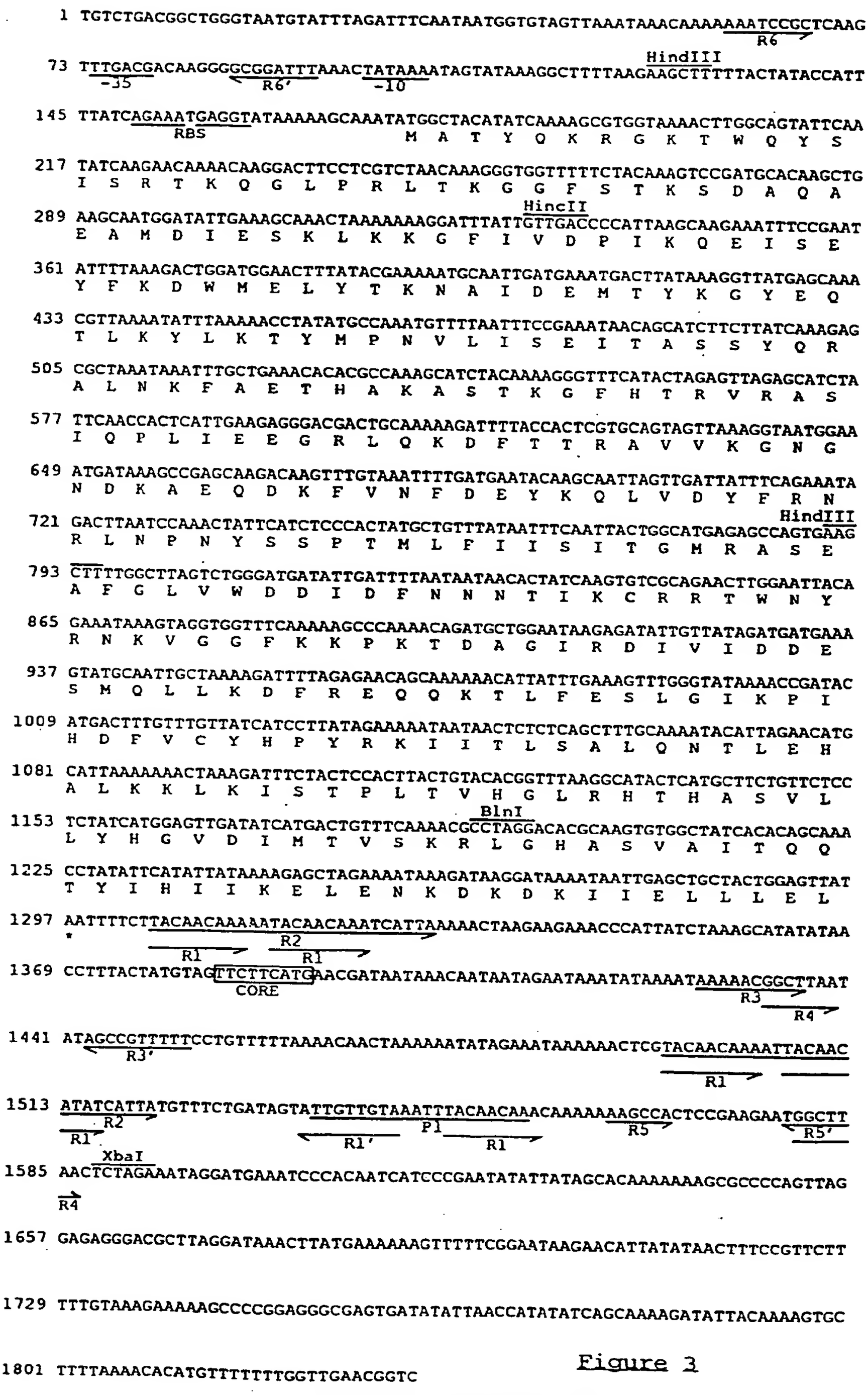

Figure 3

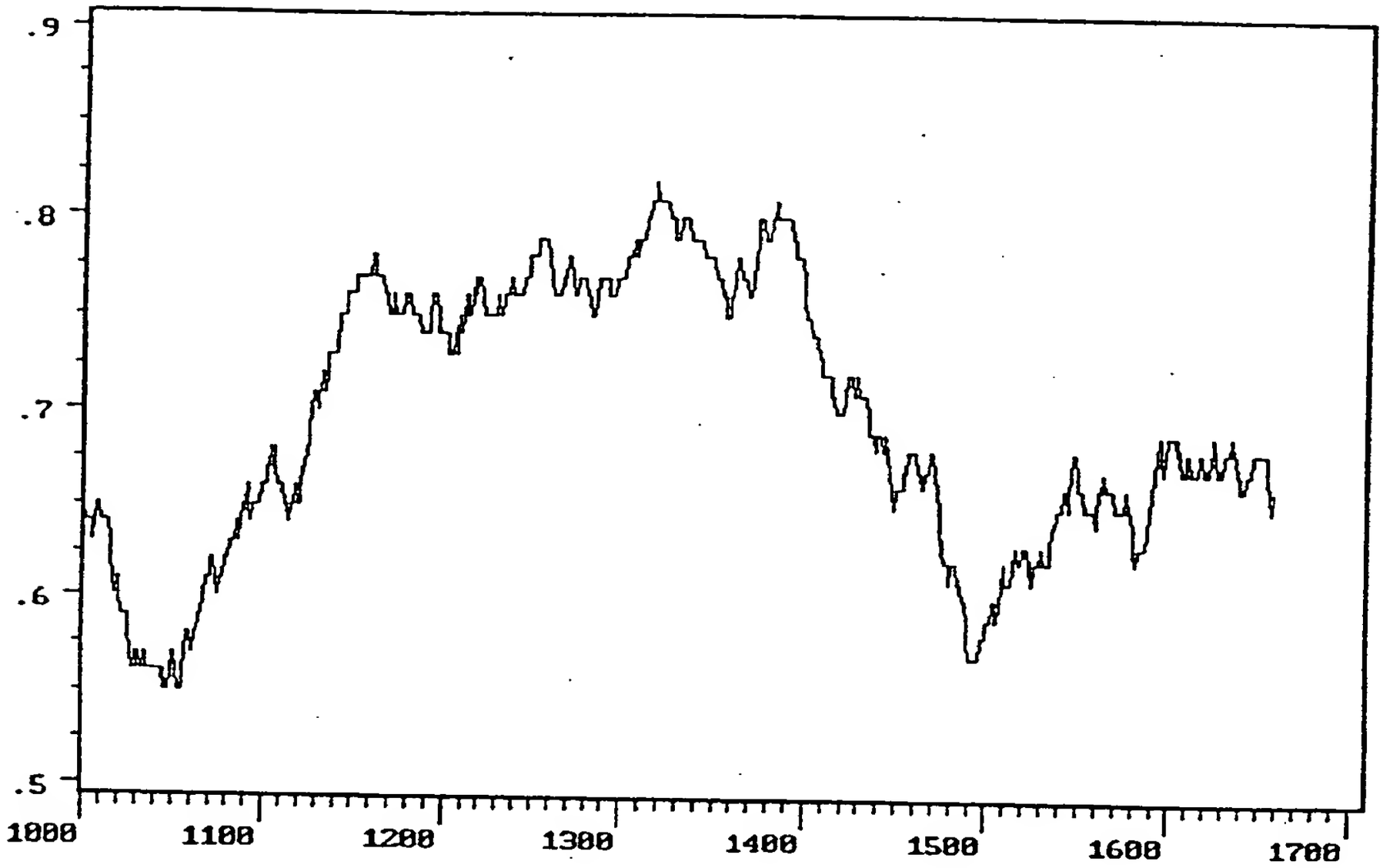

Figure 4

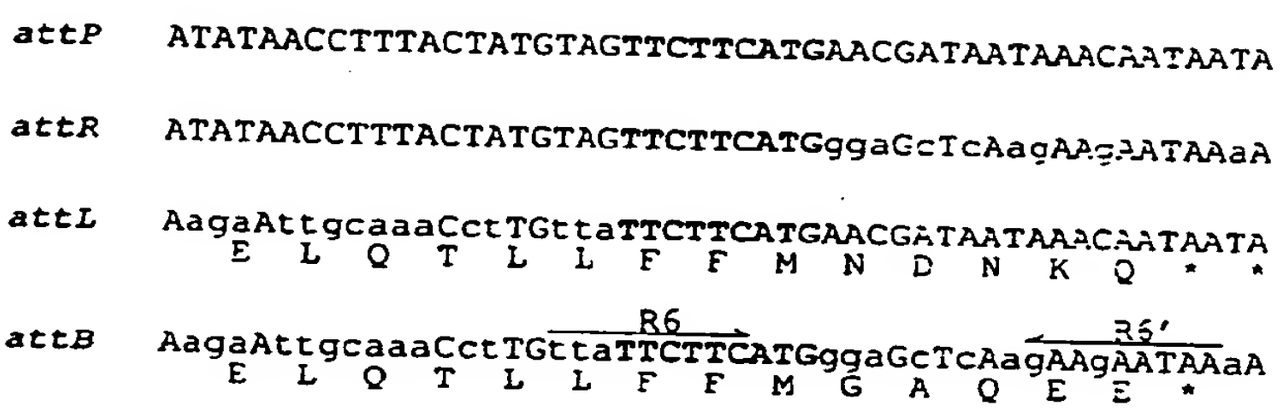

Figure 5

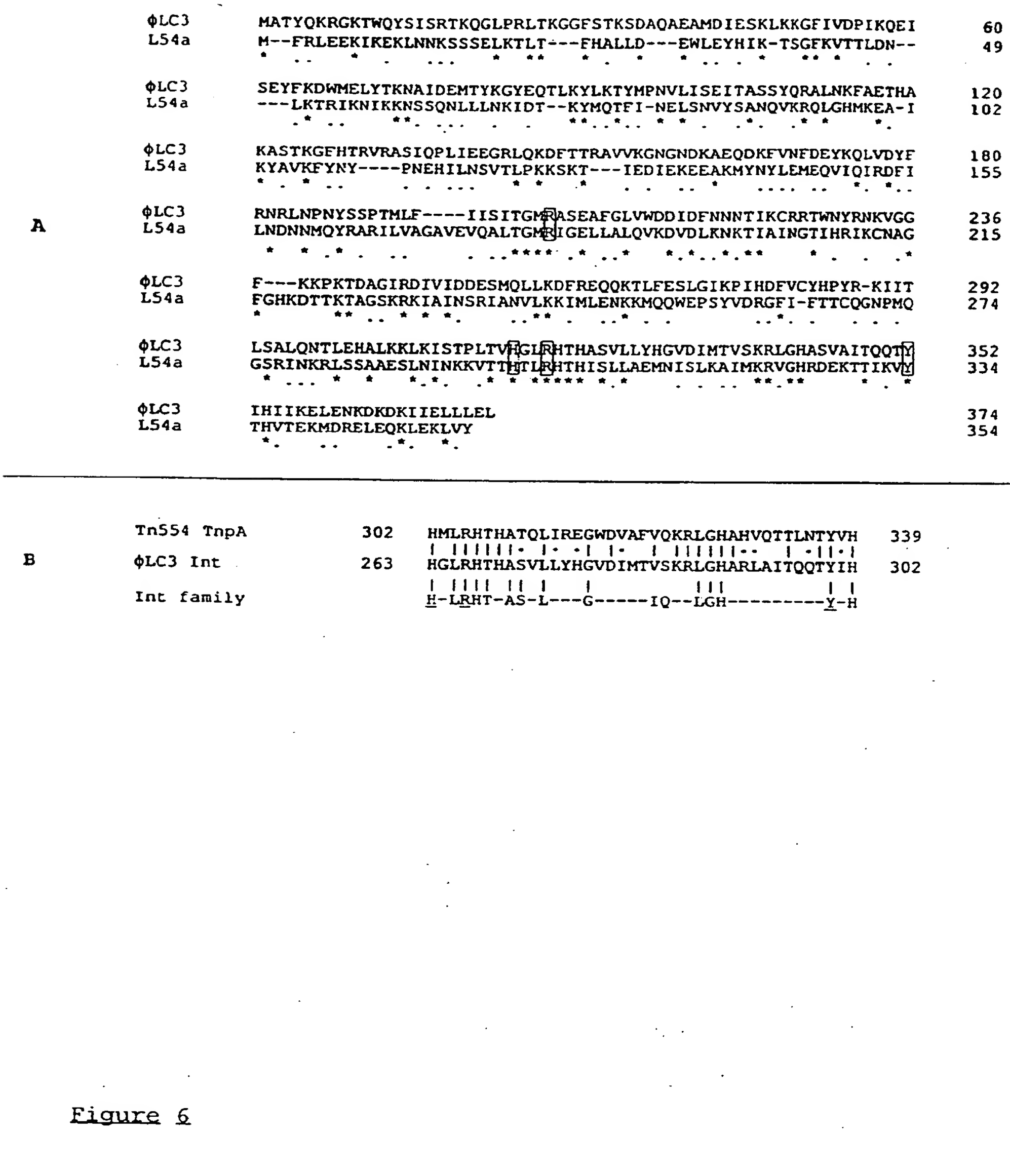

Figure 6

```
ttgccacaat actatcgact tttcaaaagt ctagtcgaag agaatgtgac acgcacaaat
tcacaactta tttctgaaaa aattggggta gatgctgcca caattcgtcg tgatttttct
ctctttgggg aattaggacg acgaggctac ggatatgaaa caaaagttct tcgtgatttc
tttggagaac tccttggaca agatcaagaa actcatatcg cattgattgg agtcggaaac
cttggacgtg cgcttcttca ttatcaattt caagacagaa ataaaatgag aattactcag
gcctacgata tttctggaaa tccactagtt ggaactcaaa cagatgatgg cattccaatt
tataatattt ctgatttaga gaagaatgtt aagaaatcag acataaaaac agccattcta
tctgttcgta aggaaaacgc tcaggaagtg gttgataggg ttgttaaagc aggaatcaaa
ggctttctta actttgcacc tattcgcttg aaagtccctt cagatgttgt tgttcaatct
attgatttaa ctaaagaatt gcaaaccttg ttattcttca tgggagctca agaagaataa
aagacaagca aaattttatc aaaactatta atgataccta ggccttcgtg tgcttaggta
ttttatttttc aagccatcat cattttaatt ttttcacaaa ttttttagttt attatttagt
```

Figure 7

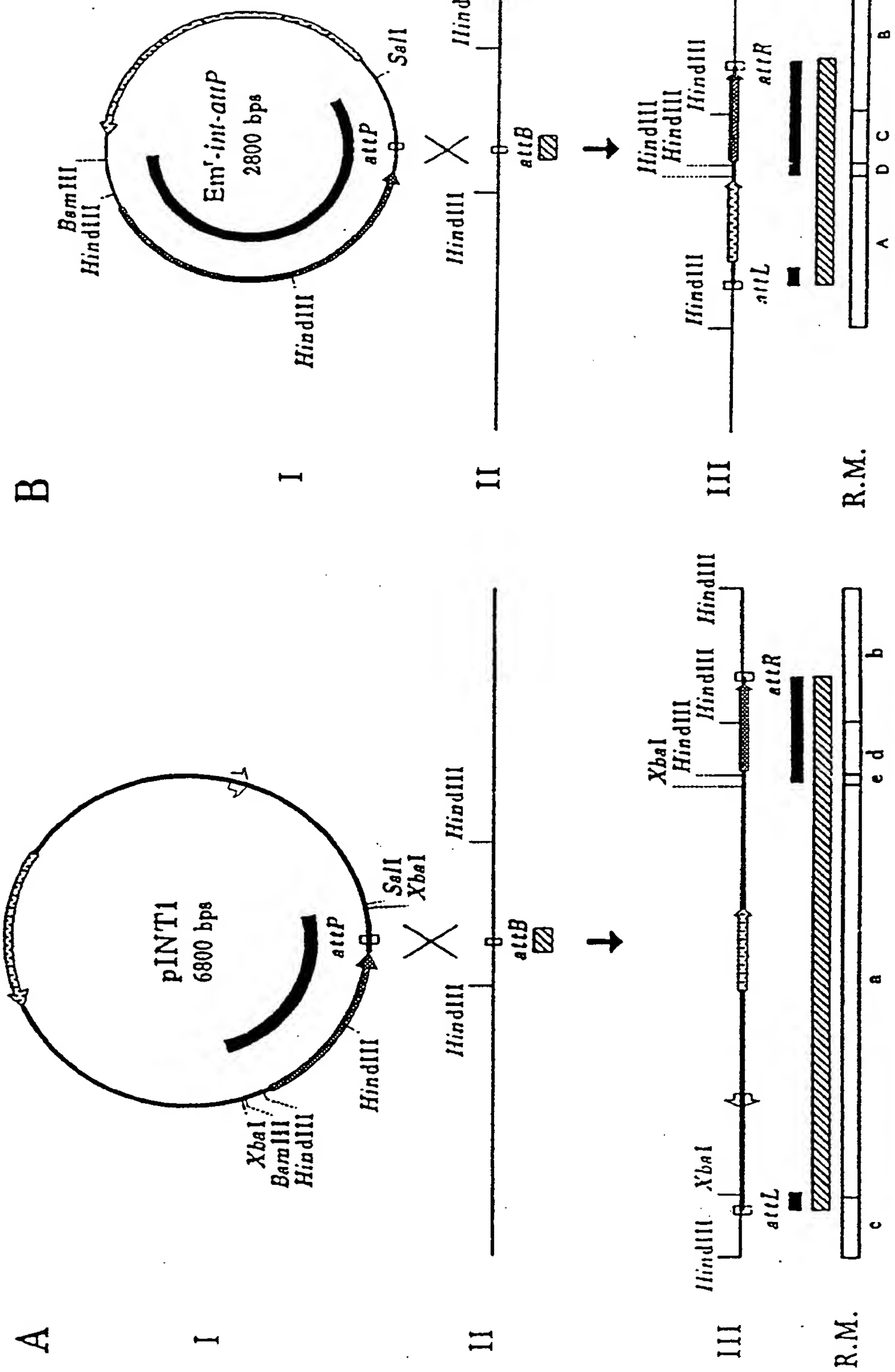

Figure 8

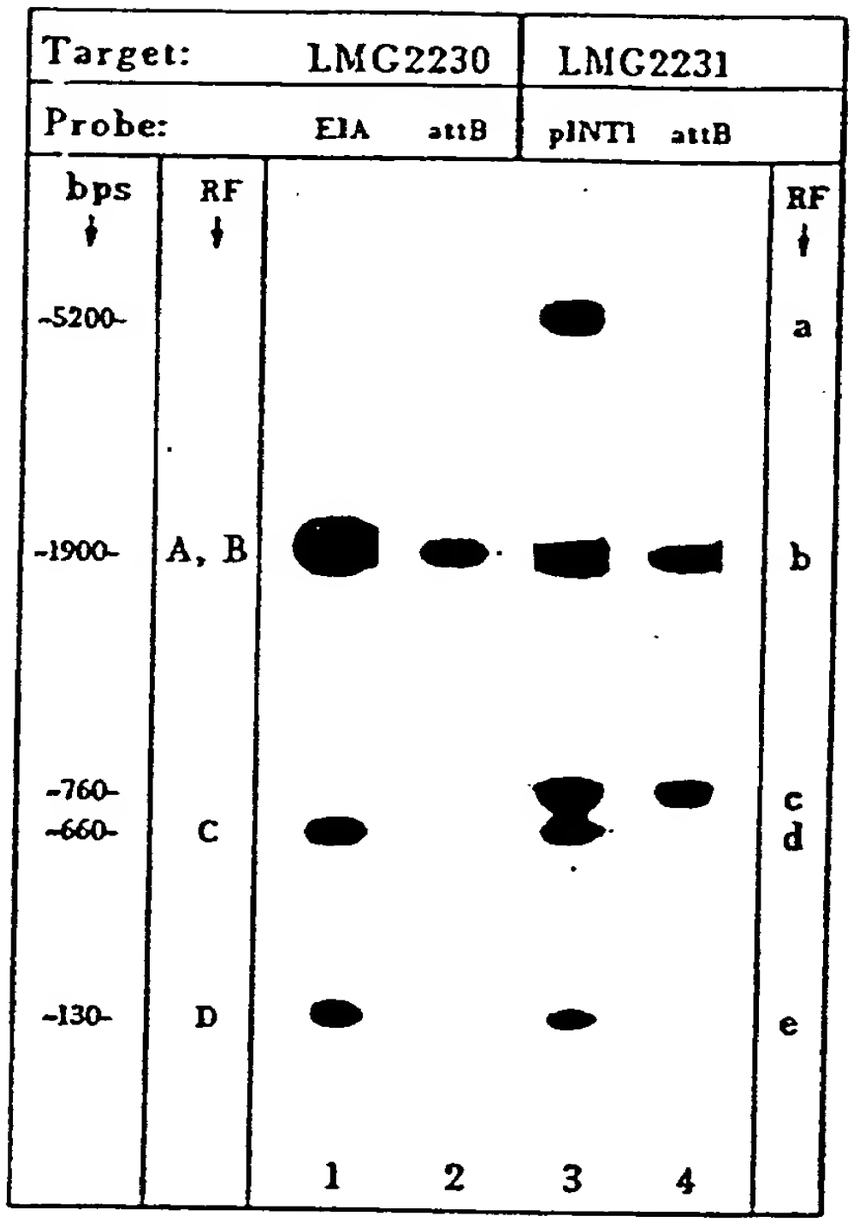
Target:
LMG2230
LMG2231
Probe:
EIA attB
pINT1 attB
bps
RF
RF
-5200-
a
-1900-
A, B
b
-760-
c
-660-
C
d
-130-
D
e
1
2
3
4

Figure 9

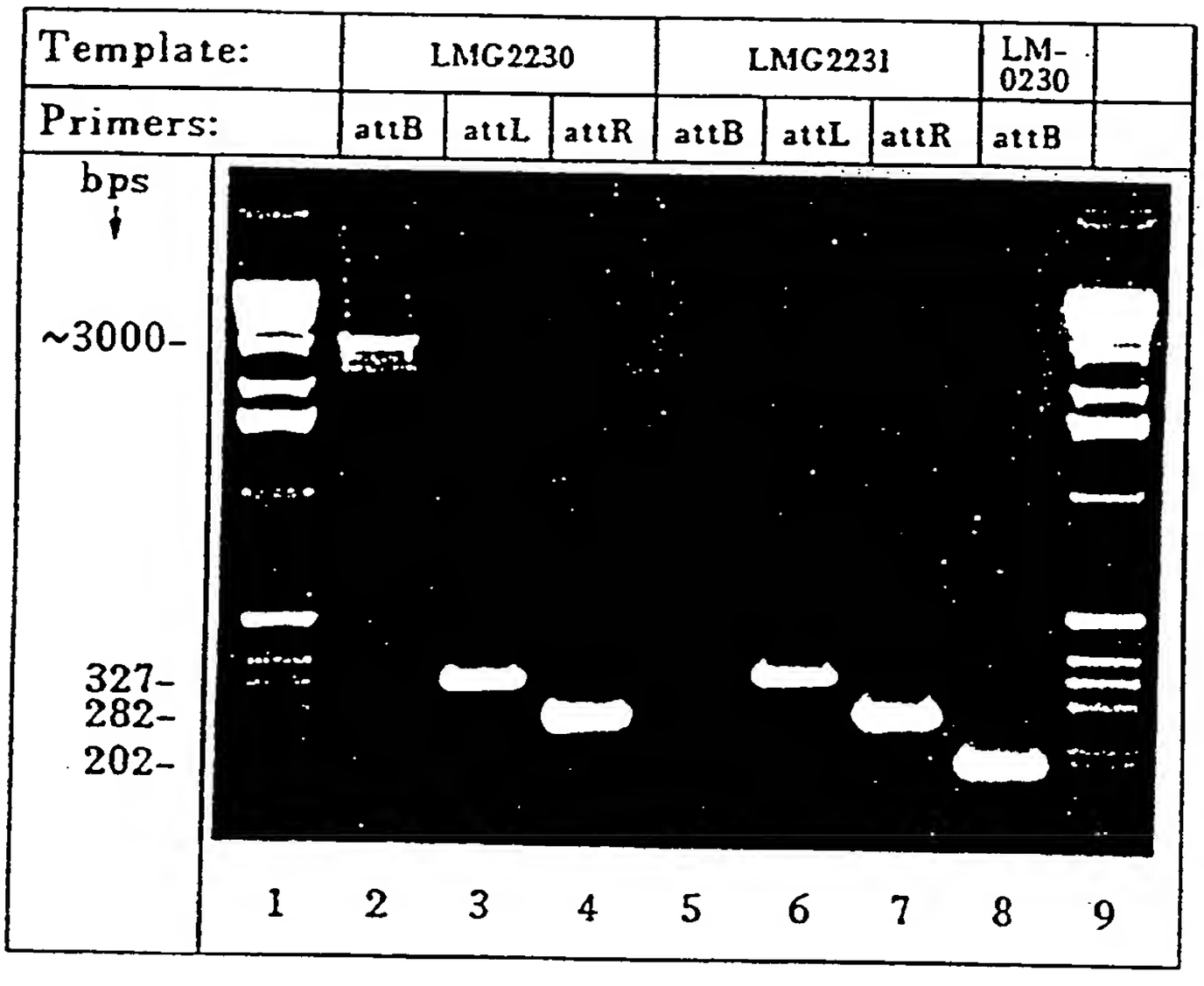

Figure 10

INTERNATIONAL SEARCH REPORT

Int ional Application No
PCT/GB 94/00365

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12N15/11 C12N15/74 C12N15/77 C12N1/21 C12N7/01

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	APPLIED AND ENVIRONMENTAL MICROBIOLOGY vol. 57, no. 11 , November 1991 pages 3206 - 3211 DAG LILLEHAUG ET AL. 'Characterization of Phi LC3, a Lactococcus lactis subsp. cremoris temperate bacteriophage with cohesive single-stranded DNA ends' cited in the application see page 3206, left column, paragraph 2 - right column, paragraph 1 see page 3210, right column, paragraph 2 --- -/--	1,3,7, 18,23

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 June 1994	[illegible]

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Montero Lopez, B

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Int ional Application No
PCT/GB 94/00365

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JOURNAL OF BACTERIOLOGY vol. 174, no. 17 , September 1992 pages 5584 - 5592 R.R.RAYA ET AL. 'Site-specific integration of the temperate bacteriophage Phi adh into the Lactobacillus gasseri chromosome and molecular characterization of the phage (attP) and bacterial (attB) attachment sites' cited in the application see abstract see page 5584, left column, paragraph 3 - right column, paragraph 1 see page 5589, right column, paragraph 2 see page 5590, right column, paragraph 2 - page 5591, left column, paragraph 1	1-23
P,X	APPLIED AND ENVIRONMENTAL MICROBIOLOGY vol. 59, no. 6 , June 1993 pages 1966 - 1968 NILS-KARE BIRKELAND ET AL. 'Transduction of a plasmid carrying the cohesive end region from Lactococcus lactis bacteriophage Phi LC3' see abstract see page 1966, left column, paragraph 2 - right column, paragraph 1 see page 1967, right column, paragraph 3	7,8,10, 13,14,23

Form PCT/ISA/218 (continuation of second sheet) (July 1992)